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DEPARTMENT OF PHARMACOGNOSY AND NATURAL PRODUCTS CHEMISTRY

PhD THESIS

Implementation of green extraction and isolation methodologies for the recovery of bioactive compounds from olive-growing products and by-products

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List of publications, posters and oral communications in congresses

- Development of a green extraction procedure with super/subcritical fluids to produce extracts enriched in oleuropein from olive leaves
  Journal of Supercritical fluids, 2012
- Design optimization study for yield, oleuropein content and antioxidant activity of olive leaves extracts obtained with Pressurized Liquid Extraction
  Journal of separation and Purification technology, 2013
- Development of an efficacious viable procedure for the recovery of hydroxytyrosol from table olive processing wastewater using absorption resin technology and hydrostatic countercurrent chromatography
  Planta medica, 2014 (accepted, in press)
- A single-step isolation of squalene from olive oil deodorizer distillates by non-aqueous hydrostatic countercurrent chromatography,
  Journal of Chromatography A, 2014 (accepted, in press)
- Integration of eco-extraction and by-products valorization in the development of olive extracts for the market of nutraceuticals: Insights from the industry
  Lecture given at Olitec European Project Workshop, Athens 2013
- A single-step isolation of squalene from olive oil deodorizer distillates by non-aqueous hydrostatic countercurrent chromatography
  Poster and flash oral presentation at the 8th International Conference on Countercurrent Chromatography, London 2014
- Design optimization study of oleuropein content and antioxidant activity of olive leaves extracts with environmentally friendly extraction techniques
  Lecture given at 1st congress on Pharmaceutical sciences, Athens 2012
- Valorization of olive industry by-products for tracing and extraction of hydroxytyrosol using various green extraction technologies
  International Congress on Green extraction of natural products, Avignon 2013
- Production of extracts from olive by-products and evaluation of their cytotoxic/cytostatic activity
  International congress on natural products research, New York 2012
- Design optimization study of oleuropein content and antioxidant activity of olive leaves extracts with environmentally friendly extraction techniques
  13th European Meeting on Supercritical Fluids, Hague 2011

Preface

“Εάν αποσυνθέσεις την Ελλάδα, στο τέλος θα δεις να σου απομένουν μια ελιά, ένα αμπέλι κι ένα καράβι. Που σημαίνει: με άλλα τόσα την ξαναφτιάχνεις”.

Οδυσσέας Ελύτης

“If you could dismantle Greece, at the end all you could see remaining it would be an olive tree, a vineyard and a boat. Which means: you can synthesize it with as much”.

Odysseas Elytis

Olive-growing in Greece and the Mediterranean is older than 5000 years old. Apart from table olives and olive oil, many other products that occur from olive growing and processing play an important functional as well as symbolic role in the lives of people. Green soap, cosmetic products based on olive oil and olive kernel, olive leaf decoctions for various diseases, olive oil as such, olive paste, heating pellet fuels and various other products have played an important role in social life since the antiquity. There is an old saying that claims you can do anything out of the olive tree. This explains why there is such a multi-level processing regarding the various parts of this valuable tree and its products. And it is not yet well explored and exploited the full spectrum of what man can recover from it.

Green chemistry in food, cosmetics and pharmaceuticals on the other hand, is more than a trend. Necessity gave birth to it, following the continuous deterioration of the environment due to heavy loads of chemicals, requiring a specific treatment for the protection of the aquifer and resulting in products that are potentially harmful. Green chemistry approaches are based on certain significant pillars:

- ✓ Lower toxic solvent usage and solvent recycling
- ✓ Lower energy usage
- ✓ Lower waste production and higher valorization

- ✓ Use of recyclable and renewable materials

The effort to integrate all of the above principles in a single process led to the development of techniques and methods that result products respecting man and the environment.

Aim of the thesis

This thesis is an effort to contribute in raising knowledge for the obtention of the totality of olive's essential by means of sustainable separation methodologies. In part A, facts that cover the olive tree and the activities of olive-growing and processing are discussed, along with matters concerning the chemistry of olive natural compounds. On the other hand, green extraction and purification technologies that have been implemented during this thesis are introduced. In part B, the scope of the experimental works of this thesis is described, while the experimental strategy and personal results are presented. This experimental part is structured in respect of the four scientific publications (two accepted and two pending) that arise during this thesis. Nevertheless, more efforts and results are presented and not only the ones that were included in the publications.

The main aim of the studies that compose the work of this thesis was principally one: To develop viable processes utilizing green extraction and purification methodologies, in order to valorize olive by-products by recovering natural products of known bioactivity, either in the form of enriched extracts or as pure compounds. Thus, the first two studies deal with the valorization of olive leaves in order to recover oleuropein, while the third study focuses on recovery of hydroxytyrosol and tyrosol from table olive process wastewater and the last one refers to the recovery of squalene from olive and olive kernel oil refining by-products.

Executive Summary

The experimental work of this PhD thesis has been scheduled on a three-axis base. The aims that were set, they concerned the optimal exploitation of four different by-products of

the table olive, olive oil and olive kernel oil industries, for the recovery of specific high added-value compounds with well-known biological properties. Those natural products were desired either in the form of enriched fractions or pure compounds. The prerequisite was to utilize as eco-friendly technologies as possible for extraction and purification and to optimize the processes in laboratory as well as in pilot scale. Thus, trustworthy estimation of industrial feasibility is provided. The main technologies that were implicated for the recovery of the target compounds are:

- Supercritical fluid extraction
- Pressurized liquid (and subcritical water) extraction
- Liquid/liquid extraction
- Adsorption resin technology
- Centrifugal Partition Chromatography

The by-products that were the subject of the tracing/ extraction/ enrichment/ identification/ quantification on studies for this PhD thesis were the following:

- Olive leaves (by-products of the pruning of the trees and of the olive fruit harvest)
- Olive mill pomace waste (biphasic process)
- Table olive process wastewater (by-product of the debittering process for the production of table olives)
- Olive kernel oil deodorization distillates (by-product of the refining process for the production of olive kernel oil from olive pomace)

The natural compounds that were targeted for their recovery, enrichment and isolation due to their well-established (and still under research) biological properties and their application in pharmaceutical, phytotherapeutical, nutraceutical, cosmetic and cosmeceutical industries were respectively:

- Oleuropein
- Hydroxytyrosol
- Squalene

In all cases, not only biomarkers' content but also the overall chemical profile of the extracts was studied. The applied scientific interest of this work was that novel green

extraction technologies and combinatorial processes as well as experimental design were implemented in lab and pilot scale for the exploitation of certain by-products that occur in very large quantities every year as a result of the agricultural/ industrial processing of the olive tree, with the aim of recovering high added value natural products.

PART A – THEORETICAL PART

1) Olea europaea (Oleaceae)

1.1) Scientific classification & Botanical description

1.1.1) Taxonomy

The olive tree (Olea europaea L.) is one of the most important representatives of the Oleaceae family. This is a family that consists of about 30 genera and 600 species . Specifically, the taxonomic classification is presented in the following table:

Kingdom:	**Green Plants**
Subkingdom:	Tracheobionata - vascular plants
Superdivision:	Spermatophyta - seed plants
Division:	Magnoliophyta - flowering plants
Class:	Magnoliopsida - Dicotyledons
SubClass:	Asteridae
Order:	Scrophulariales or Lamiales
Family:	Oleaceae - ash, privet, lilac and olives
Genus:	Olea
Species:	Europaea

Table 1. Systematic Classification of olive

The genus Olea comprises 30 species of which the only one cultivated is the olive tree (O. europaea L.). In Greece, to the subspecies *O. europaea* subsp. *europaea* coexist two varieties, *O. europaea* subsp. *europaea* var. *sylvestris* or wild olive and *O. europaea* subsp. *europaea* var. *europaea*, the commonly known to us cultivated olive tree.

Figure 1. Olive groves in Peloponnese

1.1.2) Botanical Description

The cultivated species of O. europaea is a perennial, evergreen tree reaching up to 15 meters-high. The trunk is gnarled and twisted. The leaves are lanceolate, opposite and leathery, of a silver green color on the bottom and dark green on top. The fruit is a small drupe, oval, whereas the variety of wild olive has a smaller size and still bears thinner wrist compared to that of cultivated varieties.

The small white, feathery flowers, with ten-cleft calyx and corolla, two stamens and bifid stigma, are borne generally on the previous year's wood, in racemes springing from the axils of the leaves. The development of the pollen is often hampered by various constraints. These constraints are associated mostly with chemical reactions that take place and cause a type of "incompatibility" between the reproductive parts of the plant. For this reason, the proliferation of such species and other plants of the class is asexual. This is achieved by seed, cuttings, runners, stolons or spheroblasts as well as with the aid of air or pollinators [Cronquist et al. 1981, James D. Mauseth 2006].

Figure 2: Olive botanical parts

1.2) Historical elements

In Greece, olive tree has been known and cultivated since the antiquity. It is often reported in mythology and historical texts. In Homer's Odyssey, Odysseus crawls beneath two shoots of olive that grow from a single stock, and in the Iliad, is a metaphoric description of a lone olive tree in the mountains, by a spring [Homer, Odyssey and Iliad]; the Greeks observed that the olive rarely thrives at a distance from the sea, which in Greece invariably means up mountain slopes. Greek myth attributed to the primordial culture-hero Aristaeus the understanding of olive husbandry, along with cheese-making and bee-keeping. Olive was one of the woods used to fashion the most primitive Greek cult figures, called xoana (ξόανα), referring to their wooden material; they were reverently preserved for centuries. It was purely a matter of local pride that the Athenians claimed that the olive grew first in Athens. In an archaic Athenian foundation myth, Athena won the patronship of Attica from Poseidon with the gift of the olive. Though, according to the 4th-century BC father of botany, Theophrastus, olive trees ordinarily attained an age of about 200 years [Theophrastus, On the Causes of Plants, 4.13.5.] he mentions that the very olive tree of Athena still grew on the Acropolis; it was still to be seen there in the 2nd century AD; and when Pausanias was shown it, c. 170 AD, he reported "Legend also says that when the Persians fired Athens the olive was burnt down, but on the very day it was burnt it grew again to the height of two cubits [Pausanias, Description of Greece, 1. 27. 1.]. Indeed, olive suckers sprout readily from the stump, and the great age of some existing olive trees shows that it was perfectly possible that the olive tree of the Acropolis dated to the Bronze Age. The olive was sacred to Athena and appeared on the Athenian coinage. Theophrastus, in On the Nature of Plants, does not give as systematic and detailed an account of olive husbandry as he does of the vine, but he makes clear (in 1.16.10) that the cultivated olive must be vegetatively propagated; indeed, the pits give rise to thorny, wild-type olives, spread far and wide by birds. Theophrastus reports how the bearing olive can be grafted on the wild olive, for which the Greeks had a separate name, kotinos [Isager and Skydsgaard 1992, p. 35].

The olive tree, Olea europaea, has been cultivated for olive oil, fine wood, olive leaf, and the olive fruit. The earliest evidence for the domestication of olives comes from the chalcolithic Period archaeological site of Teleilat Ghassul in what is today

modern Jordan. Farmers in ancient times believed that olive trees would not grow well if planted more than a certain distance from the sea; Theophrastus gives 300stadia (55.6 km or 34.5 mi) as the limit. Modern experience does not always confirm this, and, though showing a preference for the coast, they have long been grown further inland in some areas with suitable climates, particularly in the southwestern Mediterranean (Iberia, northwest Africa) where winters are mild.

1.3) Cultivation and consumption of olives and olive oil

Mediterranean areas cover 95% of global olive oil production. The global oil production is more than 2.000.000 tn/year. More than 30 olive varieties are cultivated in Greece, which are used for the production of table olives and olive oil. Today in Greece there are more than 91 million trees covering an area of about 6 million acres. 2.800 mills operate, 26 refineries, 50 olive kernel oil mills and 200 companies for the packaging of olive oil. Some 400,000 rural families in all geographic regions are active in olive-growing. The 30% of the total cultivated area of olive trees is located in the Peloponnese and in Crete lays 22%. According to the International Olive Oil Council (IOC), Greeks consume 74 grams of olive oil on average daily, while other Mediterranean people follow: Italians consume 39 grams, Spanish 33 grams and Portuguese 22 grams. In other countries, the consumption is minimal, with the Irish to be the last of the list with just 0.32 grams. Greece consumes 66% of the produced oil and ranks third among the olive producing countries behind Spain and Italy.

Country	Production in tons (2010)[48]	Production % (2010)	Consumption (2005)[49]	Annual per capita consumption (kg)[50]
World	3,269,248	100%	100%	0.43
Spain	1,487,000	45.5%	20%	13.62
Italy	548,500	16.8%	30%	12.35
Greece	352,800	10.8%	9%	23.7
Syria	177,400	5.4%	3%	7
Morocco	169,900	5.2%	2%	11.1
Turkey	161,600	4.9%	2%	1.2
Tunisia	160,100	4.9%	2%	5
Others	111,749	3.5%	28%	1.18
Portugal	66,600	2.0%	2%	1.8
Algeria	33,600	1.0%	2%	7.1

Table 2 . The main producing and consuming countries worldwide

The annual production of olives in Greece is 3.182.204 tons, with the country being third worldwide.

Rank	Country/Region (2011)	Production (in tons)	Cultivated area (in hectares)	Yield (q/Ha)
—	World	19,845,300	9,634,576	20.598
01	Spain	7,820,060	2,330,400	29.781
02	Italy	3,182,204	1,144,420	27.806
03	Greece	2,000,000	850,000	23.529
04	Turkey	1,750,000	798,493	21.916
05	Morocco	1,415,902	597,513	22.839
06	Syria	1,095,043	684,490	15.997
07	Algeria	610,776	295,000	14.237
08	Tunisia	562,000	1,779,950	4.848
09	Egypt	459,650	52,668	87.273
10	Portugal	443,800	343,200	12.931
11	Argentina	170,000	62,498	27.200
12	Libya	139,091	216,013	6.439
13	Jordan	131,847	62,088	21.235
14	Australia	91,067	30,407	29.949
15	Lebanon	90,307	109,213	10.580
16	State of Palestine	75,530	109,213	10.580

Table 3. Main countries for olive production

As table olives are defined the "healthy fruits of certain varieties of the cultivated olive tree, which are harvested at the appropriate stage of maturity". The quality is such that when suitably treated in accordance with international standards, the table olive processing results an edible product ensuring this remains in good shape. Global production of table olives is 1,466,500 tons of which approximately 80% is produced in the Mediterranean countries and the Middle East. Europe has a share of 43%, first being Spain. U.S. production represents about 8 % (IOC , 2005) of the global production. Greece produces about 100,000 tons, mainly black olives, consumes about 25,000 tons (fourth in global consumption after Syria , Lebanon and Turkey) and the rest is exported to EU countries and especially to Third Countries. The annual consumption of table olives per person in Greece is estimated at 2.5-

3 Kg. Greece ranked third in the European Union and the fifth worldwide in terms of production. Of the 100,000 tons the largest percentage terms in black olives, with the famous Kalamata olives. However, recent years have seen a gradual shift towards the production of Spanish-style green olive, with increasing production rate currently reaching 40%. Furthermore, in recent years the cultivation of olive is expanding rapidly beyond the traditional producing countries in the Mediterranean, in almost all countries of the world with the appropriate climatic conditions. This makes year by year the international market highly competitive and therefore the need for further development of processing industries and Greek olives standardization is becoming more urgent.

1.4) Olive tree and human health

1.4.1) Health-contributing uses of *Olea europea* L., from the antiquity to recent years

Long is the history of the usage of olives, olive oil, leaves and other parts or products of the olive tree for health disease prevention purposes. In Hippocrates' Code are encountered more than sixty pharmaceutical uses of olive oil, mainly against skin diseases, but also as a contraceptive agent. Indeed olive oil, combined with wheat and wine, is the basis of Hippocratic nutrition. In "Dietary and Therapeutic", Hippocrates writes about olive oil: " The exercises in the dust and exercises with oil differ in the following: The dust is cold, the oil is hot. During the winter the oil promotes growth, because it prevents warmness to be removed from the body. During the summer, rubbing the body with oil and water soothes the body and does not let it get too hot ".

With warm olive oil were smeared women who miscarried, especially in advanced pregnancy and in cases of metrorrhagia were given a mixture of wild olive leaves boiled in vinegar. To facilitate childbirth preparation, Hippocrates suggests a preparation made with resin from *Terebinthia*, honey and olive oil and aromatic wine, which must be drunk.

Other applications of olive oil mentioned by Hippocrates was the treatment of chronic fevers, small wounds, infiltrates, such as abscesses and boils, sore gums, and to maintain the whiteness of teeth as well as an antidote in cases of light poisoning.

Plutarch in "Morals" refers to the treatment of mastitis with water and oil (υδρέλαιο).

For the ancient healing, finest of all was the olive oil from the wild olive tree , which was a little unusual and rare, and the oil from the first pressing obtained with the "soft" crushing

olives and without the interference of hot water . According to Pliny, the oil is taken first pressing of olives when they are still white (immature) , and secondly when the olive begins to change color without however having matured. The first oil is white, the second green. The second one is good for the gums and it is excellent to keep teeth white.

The inscriptions of the famous Asclepius of Levina, in Crete, where worshiped state that "God gave a direction to be placed onto the oyster shell after burn and sanded to make it smooth with oil scented with roses and mallow oil". In the sanctuary of Asclepius the oil was used in order to prepare special ointments and remedies. Finally, beverages, leaves and flowers of olive were used as cold drops for bloodshot eyes and for stomach ulcer.

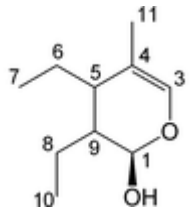
In modern times, olive and olive oil consumption has been widely discussed for the disease-preventing properties. Thus, the classical Mediterranean Diet (MD) is identified as the traditional dietary pattern found in olive-growing areas of Crete, Greece, and Southern Italy in the late 1950s and early 1960s (Trichopoulou et al., 1995). One of the most important characteristics of this diet is the presence of virgin olive oil as the principal source of energy from fat, which is a good source of monounsaturated fatty acids (MUFA) and hundreds of micronutrients. Virgin olive oil retains all the lipophilic components of the fruit, alpha-tocopherol, and phenolic compounds with strong antioxidant and anti-inflammatory properties.

Individuals who adhere to the principles of the traditional MD tend to have a longer life-span. Both men and women who report eating foods closest to the MD are about 10–20% less likely to die over the course of a study of heart disease, cancer or any other cause. The longevity of Mediterranean people has been related to olive oil, and its several microcomponents of antioxidant potential, present in all MD variants. The prevalence of the metabolic syndrome may be reduced by MD. The MD is significantly inversely associated with both systolic and diastolic blood pressure. It also has benefits in relation to the prevention of cardiovascular events, reduces the risk of mortality after myocardial infarction, and reduces peripheral arterial disease. The risk of obesity decreases with increasing adherence to the traditional MD. The MD also has a preventive effect on cancer, through its antiproliferative and pro-apoptotic effects, mostly due to the components of virgin olive oil and vegetables. There is some evidence of the benefits of the MD in relation to bone metabolism, rheumatoid arthritis, and neurodegenerative age-related diseases (cognitive deficit, Alzheimer's disease, Parkinson's disease) [Faustino et al., 2009].

1.4.2) Olive compounds with high biological significance

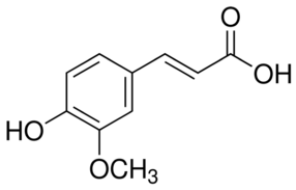
The importance of the olive products for human health is mainly attributed to the secondary metabolites that are contained in the olive oil, olives as well as the leaves. These contribute to the sensorial quality of the edible products, as well as to the beneficial effect of the so-called "Mediterranean diet" and have been used in the past for therapeutical reasons. Various secondary metabolites belonging to the wide group of phenolics and its subgroups are present in the virgin olive oil (VOO), table olives (TO) and the olive tree leaves (OTL).

Secoiridoids are compounds that are usually glycosidically bound and produced from the secondary metabolism of terpenes. The secoiridoids, found only in plants belonging to the family of Olearaceae that includes Olea europaea L., are characterised by the presence of elenolic acid in its glucosidic or aglyconic form, in their molecular structure. In particular, they are formed from a phenyl ethyl alcohol (hydroxytyrosol and tyrosol), elenolic acid and, eventually, a glucosidic residue. Oleuropein is an ester of hydroxytyrosol (3,4-DHPEA) and the elenolic acid (EA) glucoside (oleosidic skeleton common to the secoiridoid glucosides of Oleaceae) [Bendini et al., 2007]. Secoiridoids of VOO in aglyconic forms arise from glycosides in olive fruits by hydrolysis of endogenous β-glucosidases during crushing and malaxation. These newly formed substances, having amphiphilic characteristics, are partitioned between the oily layer and the vegetation water, and are more concentrated in the latter fraction because of their polar functional groups. During storage of VOO hydrolytic mechanisms that lead to release of simple phenols, such as hydroxytyrosol and tyrosol, from complex phenols as secoiridoids may be involved [Gutfinger T., 1981 and Tsimidou, 1998]. The most abundant secoiridoids of VOO, identified for the first time by Montedoro et al. (1993) and confirmed also by Angerosa et al. (1996), are the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol (p-HPEA) respectively termed 3,4-DHPEAEDA and p-HPEA-EDA, and an isomer of the oleuropein aglycon (3,4-DHPEA-EA). In 1999 another hydroxytyrosol derivative, hydroxytyrosol acetate (3,4-DHPEA-AC) was found in virgin olive oil [Brenes et al., 1999].

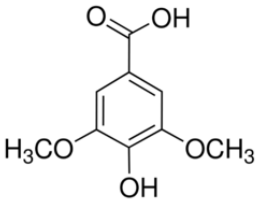


General secoiridoid skeleton

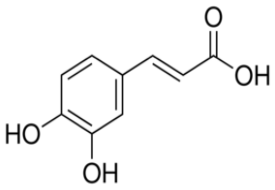
Phenolic acids are secondary aromatic plant metabolites that are widely spread throughout the plant kingdom. These naturally occurring phenolic acids contains two distinguishing constitutive carbon frameworks, namely the hydroxycinnamic and hydroxybenzoic structures. Elucidation of their roles in plant life is only one of the many ongoing investigations regarding phenolic acids: one vast area of interest lies in food quality. Phenolic acids have been associated with color and sensory qualities, as well as with the health-related and antioxidant properties of foods. One impetus for analytical investigations has been the role of phenolics in the organoleptic properties (flavor, astringency, and hardness) of foods. Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruit and vegetables, against diseases that may be related to oxidative damage (coronary heart disease, stroke and cancers) [Robbins et al., 2003 and Gomez et al., 2003]. In particular, several phenolic acids such as gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, syringic, p- and o-coumaric, ferulic and cinnamic acid have been identified and quantified in VOO (in quantities lower than 1 mg of analyte kg^{-1} of olive oil).



Cinnamic acid



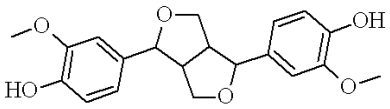
Syringic acid



Caffeic acid

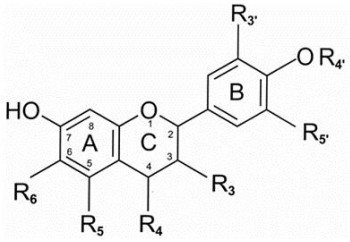
(+)-Pinoresinol is a common component of the lignan fraction of several plants such as Forsythia species and Sesamum indicum seeds, whereas (+)-1-acetoxypinoresinol and (+)-1-hydroxypinoresinol and their respective glucosides have been detected in the bark of the olive tree (Olea europaea L.). According to Owen et al. (2000), the quantity of lignans in VOO may be up to 100 mg kg^{-1}, but as with the simple phenols, considerable inter-oil

variation exists. As suggested by Brenes et al. (2002), the amount of lignans may be used as varietal marker, and they reported a method to authenticate VOO produced by Picual olives based on the very low content of the lignan (+)-1- acetoxypinoresinol in these oils.

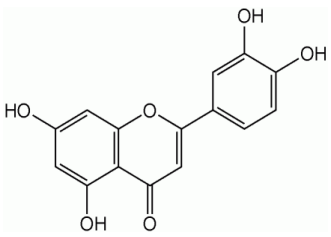


(+)-Pinoresinol

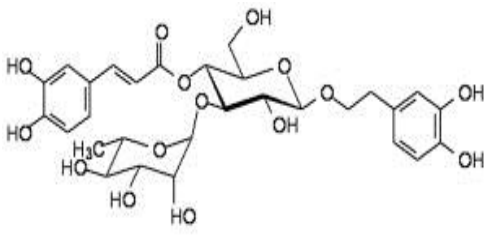
Flavonoids are widespread secondary plant metabolites. During the past decade, an increasing number of publications on the health beneficial effects of flavonoids have appeared, such those related to cancer and coronary heart diseases [Le March, 2002, Kanadaswami et al., 2005, Steinberg et al., 2003]. Flavonoids are largely planar molecules and their structural variation comes in part from the pattern of modification by hydroxylation, methoxylation, prenylation, or glycosylation. Flavonoid aglycones are subdivided into flavones, flavonols, flavanones, and flavanols depending upon the presence of a carbonyl carbon at C-4, an OH group at C-3, a saturated single bond between C-2 and C-3, and a combination of no carbonyl at C-4 with an OH group at C-3, respectively. Several authors have reported that flavonoids such as luteolin and apigenin are also phenolic components of VOO [Rovellini et al., 1997, Brenes et al., 1999,]. Luteolin may originate from rutin or luteolin-7- glucoside, and apigenin from apigenin glucosides. There are also several interesting studies in which several flavonoids have been found in olive leaves and fruits [Bouaziz et al., 2005].



General structure of flavonoids

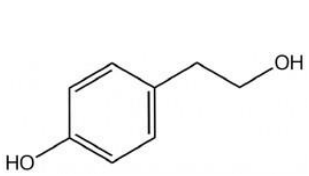


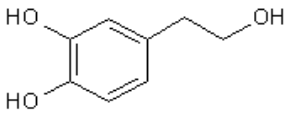
Luteolin



Acteoside

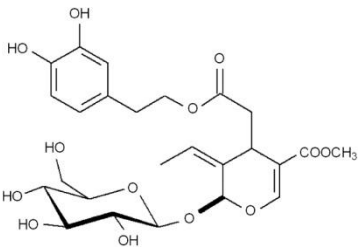
Numerous studies suggest that olive tree leaves are a significant source of bioactive phenolic compounds comparable to olive oil and fruits. It is well known that olive tree leaves contain iridoids (oleuropein as the main constituent, 11-demethyloleuropein, ligstroside etc.), flavonoids (luteolin and its glucosides, apigenin, rutin and diosmetin), triterpenes (oleanolic acid, maslinic acid etc.) and phenolic compounds (hydroxytyrosol, tyrosol, caffeic acid etc.) [Laguerre et al, 2009, Meirinhos et al., 2005, Benavente-Garcia et al., 2000, Briante et al., 2002]. The properties of the leaves have been attributed primarily to iridoids and specifically the secoiridoid oleuropein as well as hydroxytyrosol (3,4-dihydroxy-phenylethanol) [Le Tutour and Guedon, 1992; Ghisalberti, 1998].





Tyrosol Hydroxytyrosol

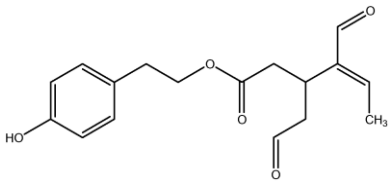
Oleuropein is also the bitter main component of olive drupe and is hydrolyzed, during treating the olives for table-olive production, to hydroxytyrosol. These two substances are known for their antioxidant properties [Owen et al., 2000], while they provide to the olive tree resistance against damage from insects and bacteria. Other natural phenols that have been identified in the olive fruit are verbascoside, ligstroside, salidroside, rutin, luteolin-7-glucoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside [Bianco et al, 2000, Briante et al., 2002].



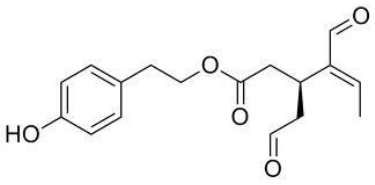
Oleuropein

Olive oil, the main source of fat of the Mediterranean diet, consists primarily of triacylglycerols rich in the monounsaturated fatty acid, oleic acid, and also contains relatively high amounts of at least 30 phenolic compounds [Miles et al., 2005]. The amount of these ‘ non-nutrients ’ or ‘ minor components ’ in the olive oil is variable, depending on

several factors, such as olive cultivar, location, climate, degree of maturation, agronomic and technological aspects of production [Oliveras-Lopez et al., 2007]. Phenols largely contribute to oil flavor and taste, the phenolic content representing a main parameter for the evaluation of olive oil quality [Oliveras-Lopez et al., 2007]. The phenolic compound (-)-decarboxymethyl ligstroside aglycone (oleocanthal) was first reported in extra virgin olive oil by Montedoro et al. [Montedoro and Servili, 1993] in 1993. A decade after its discovery, Andrewes and colleagues [Andrewes et al., 2003] reported that oleocanthal was responsible for the throat irritation and pungency elicited by some extra virgin olive oils. In 2005, Beauchamp et al. [Beauchamp et al., 2005] confirmed that the phenolic compound, oleocanthal was indeed responsible for the throat irritation elicited by extra virgin olive oils post-ingestion. Oleocanthal elicits a peppery, stinging sensation at the back of the throat similar to that of the non-steroidal anti-inflammatory drug (NSAID), ibuprofen (Beauchamp et al., 2005) and this localized irritation is due to stimulation of the transient receptor potential cation channel A1 (TRPA1) (Peyrot des Gachons et al., 2011). The perceptual similarity between oleocanthal and ibuprofen spurred the hypothesis that these two compounds may possess similar pharmacological properties.



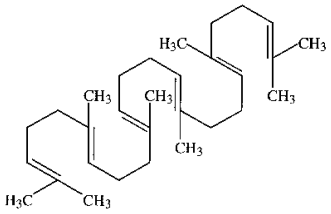
Oleocanthal



Oleacein

On the other hand, the second richest source of squalene, after shark liver, is olive oil. Indeed, some believe that the biological value of olive oil is also due to its content in squalene. Nowadays, we are convinced that in the process of refining olive oil and olive kernel oil, it is produced a waste rich in molecules with high added value, such as squalene. Indeed, squalene is the precursor for most of the remaining components of the unsaponifiable fraction of the oil [Bondioli et al. 1980]. The fact that the squalene content is higher in the extra virgin olive oil, compared with the refined olive oil [Owen et al., 2000], indicates that during the process of refining, squalene is removed from the oil. Also, the

concentration of oil in squalene depends on the variety of olives from which the oil is derived [Oueslati et al., 2009], but is usually greater compared to pomace oils.



Squalene

1.4.3) Biological properties of oleuropein, hydroxytyrosol, oleocanthal and squalene

Among the bioactive compounds of olive leaves, **oleuropein** presents very interesting pharmacological activities. More specifically, oleuropein possess several biological properties, particularly antioxidant and anti-inflammatory [Visioli et al, 2002] and has been shown to inhibit or delay the rate of growth of a range of bacteria, fungi and viruses [Ma et al., 2001]. Studies have shown that oleuropein exhibits anti-ischemic, antioxidative, hypolipidemic, antiviral and antimicrobial, antiatherogenic, cardioprotective, antihypertensive and anti-inflammatory properties. It is also known to alleviate obesity problems [Sahin et al., 2011].

Hydroxytyrosol (HT), is a phenyl ethyl alcohol, 2-(3, 4-dihydroxy-phenyl) ethanol which is best known as one of the main phenolic compounds in virgin olive oil and table olives [Blekas et al., 1995, Ghanbari et al., 2012]. It is considered as one of the most powerful naturally derived antioxidants [Ziogas et al., 2010], exhibiting a remarkable free radical scavenging activity and/or offering an indirect protection by increasing the endogenous defense systems [Deiana et al., 2007, Andreadou et al., 2010] HT has shown cardioprotective effects by preventing oxidative stress-induced endothelial dysfunction [Deiana et al., 2011], reducing oxidation of low-density lipoprotein [Castaner et al., 2011] and inhibiting lipid and protein oxidation in human plasma [Roche et al., 2009] Moreover, it has exhibited a wide range of antitumor effects, inhibiting proliferation and promoting apoptosis in several human tumor-cell lines through several mechanisms, [Fabiani et al., 2010] anti-inflammatory properties [Rossignioli et., 2013] and antiplatelet aggregation activities [Dell'Agli et al., 2002]. The last years, interest around this molecule has increased exponentially. In 2011, the European Foods Safety Authority (EFSA) issued a scientific

opinion on health claims in relation to dietary consumption of hydroxytyrosol and related polyphenol compounds from olive fruit and oil and protection of blood lipids from oxidative damage which is known to adversely affect cardiovascular health [EFSA journal 2012]. EFSA took into account that a well-designed-and-conducted study and two smaller-scale studies showed a dose dependent and significant effect of olive oil hydroxytyrosol and related compounds on lowering levels of oxidized low-density-lipoproteins (oxLDL) in blood when consumed for three weeks. These studies were supported by one short-term and one acute study showing resistance of LDL to oxidation upon consumption of HT and related compounds. EFSA determined that a minimum 5 mg of HT and its derivatives in olive oil should be consumed daily to use a cardiovascular health claim. The EFSA documents further comments that the concentrations in some olive oils may be too low to allow the consumption of the 5 mg amount HT and its derivatives with a regular and balanced diet.

Research conducted by Beauchamp and colleagues (Beauchamp et al., 2005) demonstrated that **oleocanthal** inhibits cyclooxygenase (COX) enzymes in a dose-dependent manner, mimicking the anti-inflammatory action exerted by ibuprofen. Cyclooxygenase 1 and 2 (COX 1 and COX 2) enzymes are responsible for the conversion of arachidonic acid to prostaglandins and thromboxane which are produced in response to inflammatory or toxic stimuli. COX 1 and COX 2 can be harmful to the body. In particular, COX 2 has been implicated in the pathogenesis of several cancers in both human and animal studies (Harris et al., 2003, Boland et al., 2004, Subbaramaiah et al., 2002, Ristimäki et al., 2002), and may also play a role in atherosclerosis (Chenevard et al., 2003). The novel findings presented by Beauchamp and colleagues (Beauchamp et al., 2005) demonstrate that oleocanthal not only mimics the mode of ibuprofen action, it exhibits increased potency (compared with ibuprofen) in inhibiting COX 1 and COX 2 enzymes at equimolar concentrations. For instance, oleocanthal (25 μM) inhibited 41-57% of COX activity in comparison to ibuprofen (25 μM) which inhibited only 13-18% of COX activity. Subsequent studies have shown that oleocanthal exhibits various modes of action in reducing inflammatory-related disease, including neuro-degenerative disease (Pitt et al., 2009, Li et al., 2009), joint-degenerative disease (Iacono et al., 2010) and specific cancers (Elnagar et al., 2011).

Squalene (SQ) is a precursor to the whole family of steroids, largely used as an adjuvant in vaccines and as a skin moisturizer in cosmetics as it is one of the most common lipids produced by human skin cells. It is also the main constituent of olive oil's unsaponifiable

fraction. Olive oil content in squalene was found to be 7000mg/kg [Psomiadou and Tsimidou 1999]. It has been tested for its beneficial bioactivities including antioxidant, antitumor, and cytoprotective effects. SQ has been promoted as a singlet oxygen receiver and an important lead for future cosmeceutical researches. Its cosmeceutical potential has been extensively raised after the studies which showed that squalene existed widely in human sebum, hair fat, and other surface lipids. In addition to cosmeceutical studies, SQ is regarded as an important compound for chemoprotective activities as well as nutraceutical for maintaining health under toxic exposure. [Kwon et al., 2012, Passi et al., 2002, Auffray, 2007]. Today, the great majority of SQ in the market is isolated from shark liver oil. Recently it has become a trend for sharks to be hunted to process their liver for the purpose of making SQ health capsules. Environmental and other concerns over shark hunting have motivated the extraction of SQ from vegetable sources, or biosynthetic processes instead. Thus, olive oil has been found to be a good source of SQ, as well as rice bran and amaranth seed.

1.5) Olive drupe as a raw material for table olive and olive oil production

The olive fruit is a drupe, similar to common drupes of stone fruits (peach, apricot, cherry), and shows anatomical differences with them. It consists of the epicarp (skin), the mesocarp (flesh) and the endocarp (core). Chemical composition and organoleptic characteristics vary and this is due to: a) the relatively small content of sugars in flesh (2.5-6% compared to 12% of other drupes) and b) the increased content of oil in the fresh flesh (17-30% vs. 1.5%).

In the early stages of development of the olives and until the completion of the development of the core (mid to end of August) the oil formation is negligible. Then the pericarp is gaining in weight and dimensions and is progressively enriched in oil while the core retains its weight almost stable. The fruit ripening and therefore the olive formation are influenced by environmental factors, such as climatic conditions, soil type and exposure of the grove. High oil production occurs when the olive is grown in ideal conditions (mild climate, sunny area) and is harvested at the appropriate time.

In August-September olives are unripe, horde and have tough flesh that processes difficult. Later (October -November) some varieties become half ripe when the color of the fruit turns from green to black. The flesh is still compact, but it yields oil with excellent organoleptic properties. Between December and January the fruit is ripe and yields oil of

average quality, while in the next two months, more varieties of olives become over-ripe and shrink, losing their color.

The processing of olive fruit in order to produce oil comprises the following steps :

- ✓ Harvesting fruit - Storage
- ✓ Transfer to the mill - Defoliation
- ✓ Wash - Breaking - Grinding olives
- ✓ Malaxation of the olives - Pressing
- ✓ Separation of oil from vegetable water
- ✓ Quality control , Storage , Maintenance , Packaging

1.6) Drupe Harvest

The harvest of the fruit is carried out usually by hand or mechanically. The traditional system of harvesting is done by beating, i.e. hitting the branches of trees with sticks. The fruits fall in olive harvesting nets, which are spread around and under the trees and then collected from the ground. This method has a good yield, but results in dropping many leaves, breaking tender shoots and injures the tree. Another method is the "natural fall ", in which the fruits are harvested directly from the ground, after their natural falling in nets. This method is preferred when the trees are large scale and there are not sufficient laborers. The fruits are harvested gradually (at least once every two weeks), thus the quality of olive oil varies significantly. Another disadvantage is the prolonged harvest period (3-5 months).

The above methods have been replaced by "milking" the branches with the hands, “combing” the drupes and allowing them to fall into small baskets, which bind at the waist of each worker. This method avoids injury to the fruit but has the disadvantage of high labor costs. Harvesting by hand can be improved using motorized equipment. This consists of a telescopic shaft (length 2.5-3 m) at the end of which there is a rotating cylinder with plastic whips or an accessory with plastic fingers on the palm device. The system works mechanically, hydraulicly ,pneumaticly or electrically. The operator of the machine causes simple beating of the branches of the tree and the fruits drop onto nets. The mechanical harvesting systems have distinct economic advantages over traditional systems harvest by

hand, due to the reduction of labor costs and harvesting time. The most common mechanical harvesting system is the vibrator mounted in the tractor. The vibrator is mounted on an agricultural tractor and uses a hydraulic pump to transfer power to a vibrating head that is attached to the trunk of the tree. With this method, the whole tree is vibrated with vibrations of short duration, which result in the fall of the fruit in the olive harvesting nets initially placed under the tree. A disadvantage of this method of harvesting is the injury of the root system of the tree. After harvesting, the olives are delivered to processing plants for treatment as quickly as possible. The transfer is done in plastic crates with ventilation holes or plastic bags.

Figure 3. Olive harvesting: Manual and mechanical

1.7) Olive oil production processing steps

1.7.1) Storage

The processing of the olive fruit should be done immediately and any delay has an adverse impact on the quality of the produced oil. Theoretically the rate of collection should be such that the olives can be treated the day treated without prior storage. But this is impossible because in that case the facilities of the mills would be disproportionately large to be efficient in processing the olives the same day and complete the oil production within the

harvest time limits. The period of intensive harvesting, the quantity of olives transported to the mills daily is greater than their capacity, so the excess is stored in piles and wooden boxes and are being process another day.

1.7.2) Olive oil extraction

1.7.2.1) Processing method and variations

The olives when arriving at the mill contain inevitably other materials which may come from the same tree (leaves, twigs, bark etc...) or dust, sand, rocks, mud, etc. All these are separated from the olives by means of a sieve or with possible air stream in the so-called defoliators.

Figure 4. Deleafing equipment in a modern olive mill

Then the fruit is washed either in still water or using mechanical laundry. The water can be recycled after precipitation of the solid components or filtration. About 100 to 120 liters of water are required in order to wash 1000 kg of olives. The olive fruit after washing it is subjected to grinding or milling and then kneading in order to become a homogeneous pulp. Pulp is pressed or centrifuged resulting the olive oil.

Grinding is the first stage of processing the olives after all the above preliminary steps. This treatment affects both the quantity and quality of the oil will that will separate afterwards. In the classical type mills (presses), which are the traditional exhaust systems, olives are broken in the stone mills. These consist of one, two or three large stones of a cylindrical or

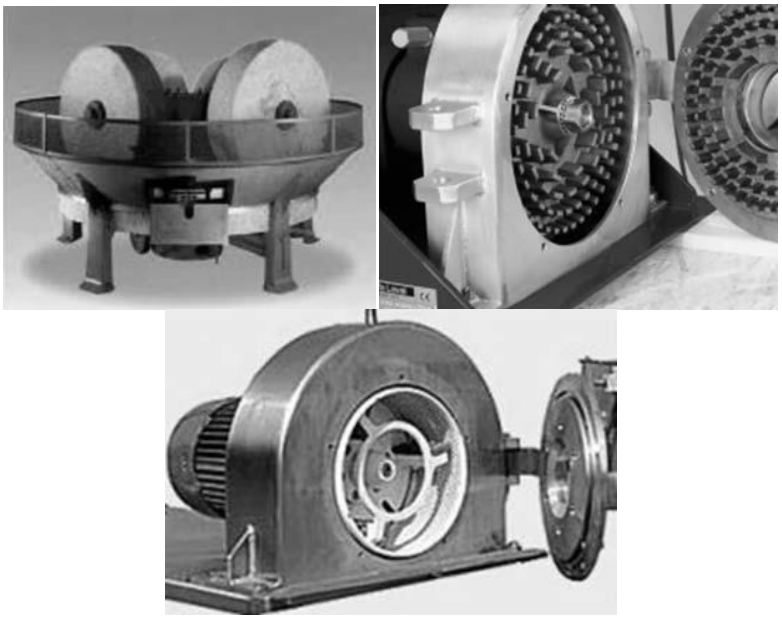
conical shape which are made of granite and revolve around a wooden or metal shaft of a similar composition, placed on a stable base.

Since the middle of the last century the stone mills began to be substituted by metal crushers and hammer mills, though they remain widespread in Greece and Spain. The metal shredders occupy minimal space, crushes the olives within a short period of time (5-10 seconds) and a closed area so as to prevent oxidation of the oil, and varying degrees of grinding the fruit are possible. They exhibit the following disadvantages though: a) They promote the formation of emulsions and are responsible for high rates of sludge formation in the oil, b) They always contaminate the oil with heavy metals and c)They do not always perform perfect work because they cannot open up all the oil-bearing cells of the flesh of the olives.

The crushing of the olives is the most basic step of processing the olives regardless of the delivery systems of the oil afterwards. It promotes to the agglomeration of small oil particles into larger drops of oil. This agglomeration is a prerequisite for separating oil from the solid paste and vegetable juice. The process is done in special malaxation apparatuses, which consist of a variable-shaped basin and capacity depending on the type of mill.

Crushing is normally carried out using a traditional stone mill or by means of a hammer or disk crusher. Characteristically the traditional stone mills have batch-processing potential whilst the mechanical crushers have varying structural features. For example, they have crushing devices that are designed and built a variety of ways to ensure continuous on-line olive processing and decanting for oil extraction. The stone mill consists of three stone rollers or wheels, which roll in circles on a slab of granite to grind the olives into a paste. In hammer crushing machines, a three- or four-lobe rotor with wear-resistant metal plates crushes the olives against a stationary grid. The diameter of the grid holes determines the thickness of the paste. Disk crushing machines, on the other hand, crush the olives between two toothed disks – one stationary and one that rotates. A new technology in virgin olive oil production is olive de-pitting. This ensures that the paste consists solely of the fleshy part of the olive (mesocarp), without the stone or pit (endocarp) that holds the seed. The de-stoner consists of a cylindrical perforated stationary grill and a rotary shaft. The olives are pushed by centrifugal force towards the perforated grill. Olive tissue crosses the grill whilst the kernel remains inside the cylinder. Using this method the grinding of pulp tissues is not

drastic. Each machine has its advantages and disadvantages. The choice of crushing system appears to depend on subjective circumstances.



Stone mill Disk crusher Hammer crusher

Figure 5: Various types of the machines employed in the crushing step

Finally, an important factor is the heating during malaxation that facilitates the removal of the oil from the plant cells, because high temperature reduces the viscosity and oil droplets move faster and are agglomerated. Nevertheless, the key is not to increase the temperature too much, because it will have adverse effects on the quality of the oil, due to the volatile components losses which result loss on the characteristic aroma and taste. The material of the surfaces of the mixer should be stainless steel to avoid contamination of the oil with heavy metals trace which catalyze oxidative reactions and accelerate the deterioration of oil during storage.

The final step of the process involves the separation of the oil from the olive paste and vegetation water. This is performed through a procedure called decantation. This is a newer technique of separating the oil from the paste and is based on the difference of specific gravity for the components of the olive paste (oil, water and solids). In centrifugal mills, the paste after the kneading mixer in vertical or horizontal arrangement, is diluted with sufficient quantities of water and then it is centrifuged through the decanter (centrifugal separator), where it is separated into three phases.

The three-stage process is a continuous process which has replaced nowadays the traditional pressing method. The ground olives are placed in a decanter, wherein different parts (olive oil, vegetation water, olive cake) separated by the effect of centrifugal force. The main disadvantage of this method is the large quantities of water needed and therefore the production of significant volumes of waste water causing pollution (olive mill wastewater). It is estimated that 1000 kg of fruit produce 500 kg pomace (moisture content 5%) and 1200 kg waste liquids.

Nevertheless, a more modern and continuous system appeared on the market in the last 30 years, the biphasic system (also called "eco- system "). In this process no additional water is added to the paste. The final products of the treatment are olive oil and olive pomace waste which incorporates the vegetation water (wet pomace). When processing 1000 Kg fruit about 800 kg of waste are produced.

After malaxation, as already mentioned, the oil is either completely free or enclosed in the form of small droplets within microgels, either end as an emulsion incorporated in the plant liquids. The greater the amount of free oil and the fewer the microgels, the easier is the extraction of oil by centrifugation (Mendoza, 1975). For the separation of oil in the paste, alone the centrifugal force is sufficient. Conversely, when it is enclosed in microgels or emulsions, this is achieved only by adding a large quantity of water, which modifies the colloidal constituents and facilitates the separation. Thus, at the end it is necessary for the final separation a pass from a final separator. This is composed of a fixed core and a movable drum which rotates at high speed. It has a large number of conical discs, properly adjusted. The liquid phase is distributed in thin layers onto the circumferential surface of each disc and so the centrifugal force becomes more effective, finally separating the oil from the waste water and solid impurities. The final separation is influenced by the specific gravity, shape and dimensions of the droplet, as well as from the viscosity and temperature. Figures 6 and 7 illustrate the flow scheme and the flowchart process for the variable olive oil processing methods.

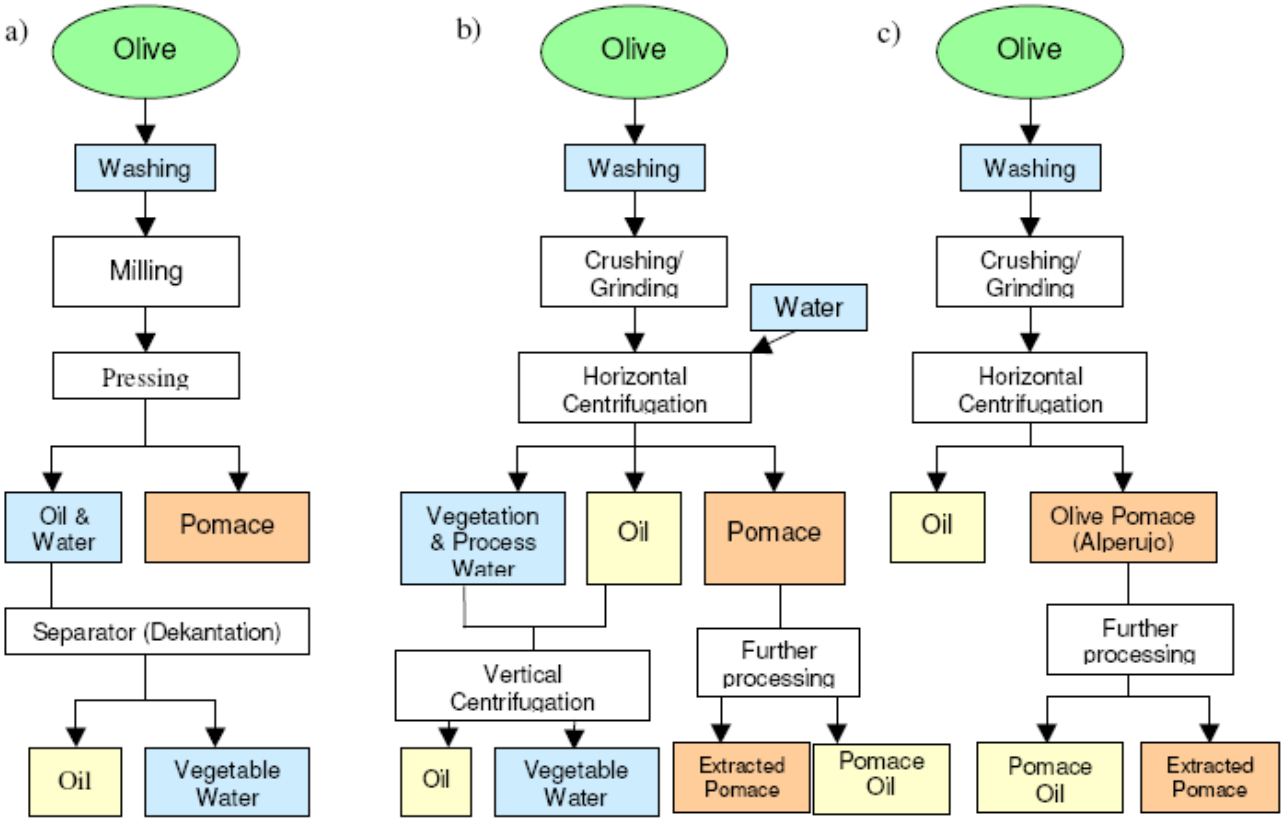


Figure 6. Flow schemes of the 3 different olive oil production processes:
a) Traditional process, b) 3-phase decanter process, c) 2-phase decanter process.

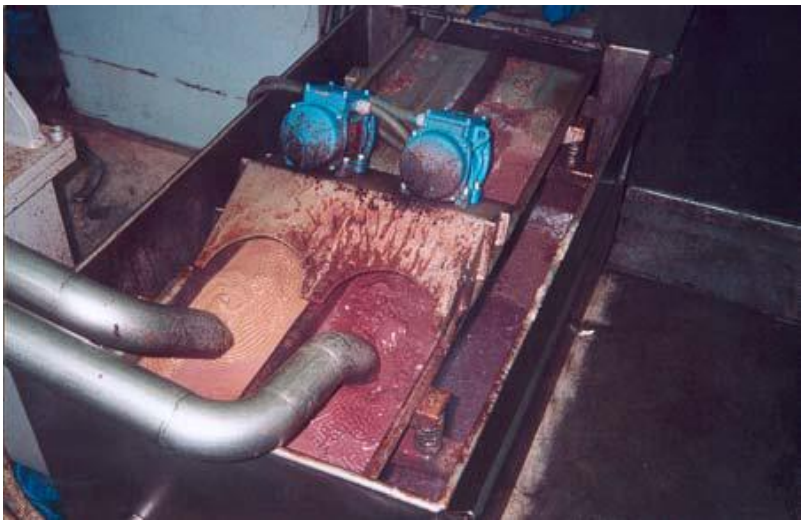


Figure 7. Olive mill wastewater

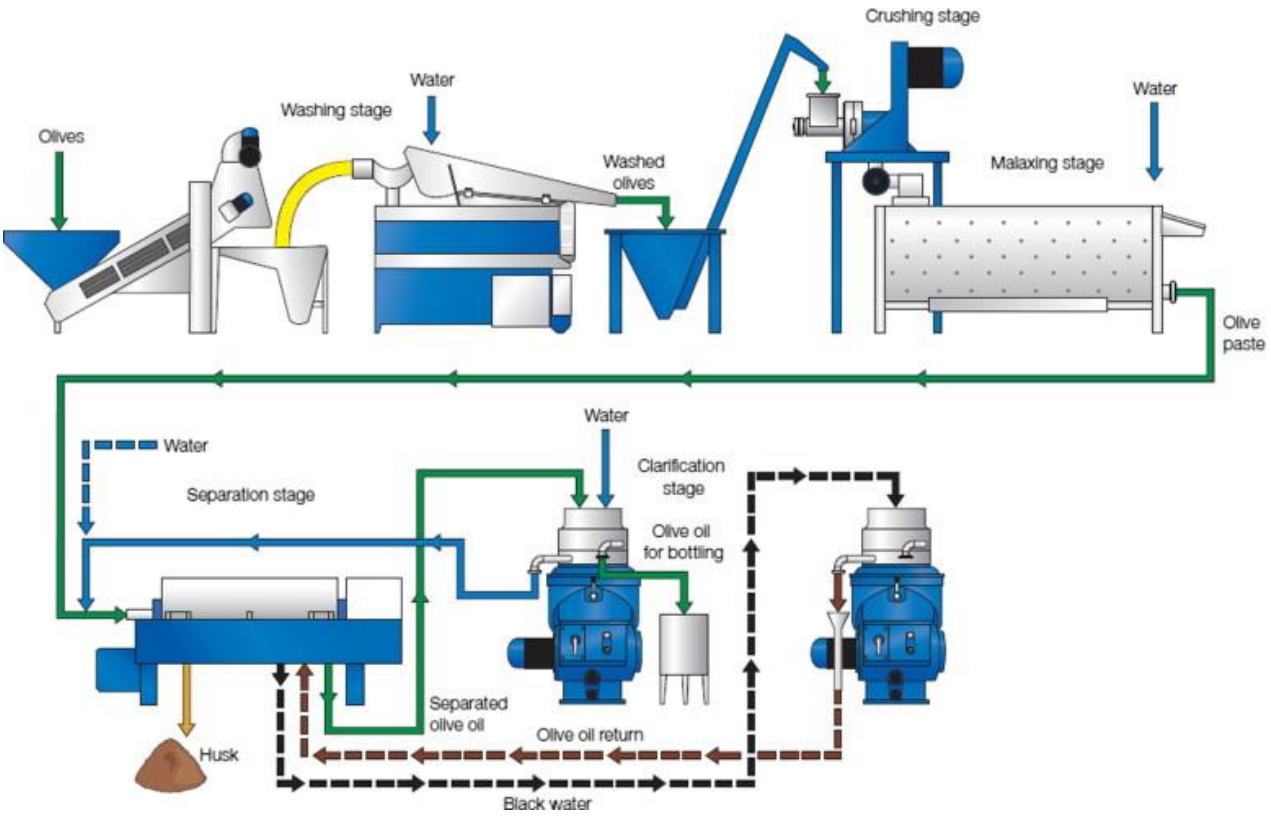


Figure 8. Flowchart of an olive processing line

1.7.2.2) Effect of processing method on olive oil characteristics

The sensory quality of virgin olive oil is strictly related to phenolic and volatile compounds. Phenolic compounds are directly responsible for any bitter and pungent taste in the oil and their concentration affects the shelf-life of the product. Several volatile compounds, particularly aliphatic alcohols, low-molecular-weight terpenes, ketones, ethers, furan and thiophene derivatives, and, most importantly, C 5, C 6 and C 9 saturated and unsaturated alcohols and aldehydes, determine the aroma of the oil. The release of phenols and the formation of volatiles are two basic components of virgin olive oil quality that directly relate to the mechanical extraction process itself. In this ambit, control of endogenous enzymes of olive fruit during processing is the most critical point in the mechanical extraction process of olive oil. In fact, the secoiridoid concentration in the virgin olive oil is largely due to the activation of the glycosidases of olive fruit that activate the formation of aglycon, while the oxidoreductases such as polyphenoloxidase (PPO) and peroxidase (POD) can catalyze their

oxidation during the oil mechanical extraction process and subsequently trigger the autoxidation mechanism. Catalano and Caponio (1996) compared the use of stone mills with hammer crushers. Their comparisons offered significant insights into the phenomena occurring in paste preparation and the subsequent interactions taking place amongst the components of the drupes. They also indicated the most rational use of either of the two machines in industrial practice. When the paste is prepared using hammer crushers the oils produced contain greater amounts of polyphenols than by preparation with a stone mill. This frequently corresponds to an increased resistance to autoxidation and causes a marked 'bitterness' and 'pungency'. This is particularly true for oils extracted from certain cultivars, which can lead to an unpleasant experience for the inexpert consumer. In order to upgrade the organoleptic quality of extra-virgin olive oil and to enhance their preservation, two systems have been suggested for processing procedures. For olives yielding oils rich in total polyphenols it is best to use the stone mill or any innovative system that reproduces the milling process such as the disk crusher. However, the hammer crusher is more suitable in processing olives yielding very 'sweet 'oils with a low content of polyphenols and that are thus not very preservable. These findings have been confirmed by Di Giovacchino et al. (2002). When olive oil mills are equipped with pressure systems, olive crushing is generally carried out by granite millstones (with 2 – 6 stones) for 20 – 30 minutes. The resulting olive paste is squeezed by a hydraulic press. This approach ensures that good oil extraction yields are obtained. When olive oil mills are equipped with centrifugation systems olive crushing is generally carried out using metallic crushers such as mobile or fixed hammers with toothed disks. These crushers have a high working capacity and exert a violent action that breaks the cells of the olive flesh containing oil. The resulting paste leads to good extraction yields after a suitable malaxation step. Olive crushing can increase the temperature of the olive paste due to the frictional energy generated by the rotating crushers. The data indicate that the crushing method, i.e. using a stone mill (gentle) or a metallic crusher (very violent), does not influence qualitative parameters such as free fatty acid percentage, peroxide value, specific spectrophotometric absorptions in the UV region or the organoleptic assessment. The crushing method, however, does have a clear influence on the total phenol content of oils. The use of the more violent metallic crushers results in an oil with a total phenol content higher than that obtained using a stone mill. This is due to the more complete disruption of olive flesh that liberates higher quantities of phenolic

substances. These then bond to the different cellular tissues of the olive flesh, and hence increase their concentration in the olive paste [Di Giovacchino et al., 2002]. Caponio and Catalano (2001) used a hammer crusher and a disk crusher in order to evaluate the effect of differing processing temperatures on the quality of the virgin olive oils obtained. The results showed that the hammer crusher produced more intense fragmentation of the olive pits than the disk crusher, resulting in a substantial increase in output temperature. Higher temperatures in the crusher during olive processing lead to a shorter shelf-life for the resulting oils. All of the analysis performed so far demonstrates that the oils obtained from hammer-crushed pastes degrade at a greater rate than those from disk-crushed pastes. The data suggest that the temperatures reached during fast olive crushing – either with traditional hammer crushers or with disk crushers – influence the quality and preservation of olive oils and that these oils are more susceptible to auto-oxidation if they are produced with a hammer crusher rather than with a disk crusher.

1.7.3) Olive oil processing wastes: Types and composition in view of valuable compounds

The manufacturing process of olive oil has undergone evolutionary changes. As it has already been mentioned, the traditional discontinuous pressing process was initially replaced by the continuous centrifugation, using a three-phase system and later on a two-phase system. Depending on the different olive oil production method there are different kinds of wastes, being mainly one type of residue or another according to the most common extraction technology used in each country.

First of all the occurring residues, are the olive leaves that are removed during the defoliating process. Olive tree leaves (OTL) gather the interest of the scientific community and the industries worldwide as their health promoting benefits are constantly being shown by an ever-increasing number of scientific data [de Castro et al., 2009]. They are considered byproducts of olive farming, one of the most important activities in the Mediterranean region, rep-resenting almost 10% of the total weight of materials arriving to the olive mill. Research into olive leaves has revealed that their health properties are attributed to a group of secondary metabolites they contain, namely biophenols that display a wealth of both structural variety and diversity of important activities [Sahn et al., 2011]. They contain phenolic compounds including flavones (luteolin-7- glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin, and diosmetin), flavonols (rutin), flavan-3-ols (catechin),

substi-tuted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid, and caffeic acid) and secoiridoids (oleuropein) [Mustafa et al., 2011]. The classic production of olive oil generates three phases and two wastes: olive oil (20 %), solid waste (30 %) and aqueous liquor (50 %). The solid waste or olive pomace waste (διφασική πυρήνα, alperujo) is a combination of olive pulp and stones. The aqueous liquor comes from the vegetation water and the soft tissues of the olive fruits, with water added during processing, so-called olive-mill waste water (OMWW) (κατσίγαρος, alpechin). The presence of large amounts of organic substances (oil, polyphenols, protein, polysaccharides, etc), responsible of the high COD values (up to 220 g/L) and minerals salts, represent a significant problem for the treatment of waste water (Borja et al., 1997; Niaounakis and Halvadakis, 2004).

During the olive oil mechanical process, the major proportion of the phenolic compounds are found in the aqueous phase, while only a minor percent (<1%) are located in the olive oil [Vierhuis et al., 2001].This explains why a large fraction of them can be found in the olive mill wastewater (κατσίγαρος, alpechin). The use of two-phase centrifugal decanters makes that, despite the obtained virgin olive oil has a greater concentration of phenolic compounds than that obtained by the three-phase mode, most of them (about 98%) remains in the olive pomace waste (διφασική πυρήνα, alperujo) [Vierhuis et al., 2001]. Therefore, both residues seem to be an affordable and abundant source of natural antioxidants. More specifically, oleocanthal has been found in very significant amounts in olive pomace waste –produced by the most modern two-phase systems- comparable to its concentration in olive oil [Cicerale et al., 2011]. The main biophenols occurring in OMW are tyrosol, hydroxytyrosol and oleuropein. Antioxidant property of ortho-diphenolic compounds of OMW, particularly of hydroxytyrosol attracts considerable attention to recover these compounds from OMW by developing an effective process. Oleuropein, which is an ester of hydroxytyrosol (3,4-DHPEA) and elenolic acid (EA) glucoside, also gains interest as a natural food antioxidant. On the other hand, intensive studies on phenolic composition of olive mill wastes have introduced novel antioxidants to olive oil sector. For instance, Obied et al. [10] recently identified two new biophenolic compounds in OMW extracts, namely hydroxytyrosyl acyclodihydroelenolate and p-coumaroyl-6’-secologanoside (comselogoside). The authors reported that antioxidant scavenging activity of these compounds was higher than hydroxytyrosol and oleuropein.

1.8) Olive and olive kernel oil refining

1.8.1) Refining process

High acidity olive oil as well as olive kernel oil which is extracted from the olive pomace and/or olive mill wastewater using an organic solvent such as n-hexane, need to refined in order to meet the criteria for consumption. Oil refining is the process that takes place for lower quality olive oils, which have an unpleasant odor and taste due to the content of free fatty acids in order to obtain characteristics similar to virgin oil. The process of refining oil basically has to do with converting non-edible oil in edible. The process involves three stages of treatment, removal of margarines and neutralization of acidity, discoloration and deodorization. The first stage is carried out by removing of the margarine and the neutralization of free fatty acids from the oil. This is done with the use of caustic soda, which has the capacity to neutralize the free fatty acids, directly affecting the quality of the oil, and create soaps. After this step, neutral oil is obtained which has then to be decolorized and deodorized. Decolorization and deodorization are the most important stages in the process of oil refining and the process flow is shown in Figure 9.

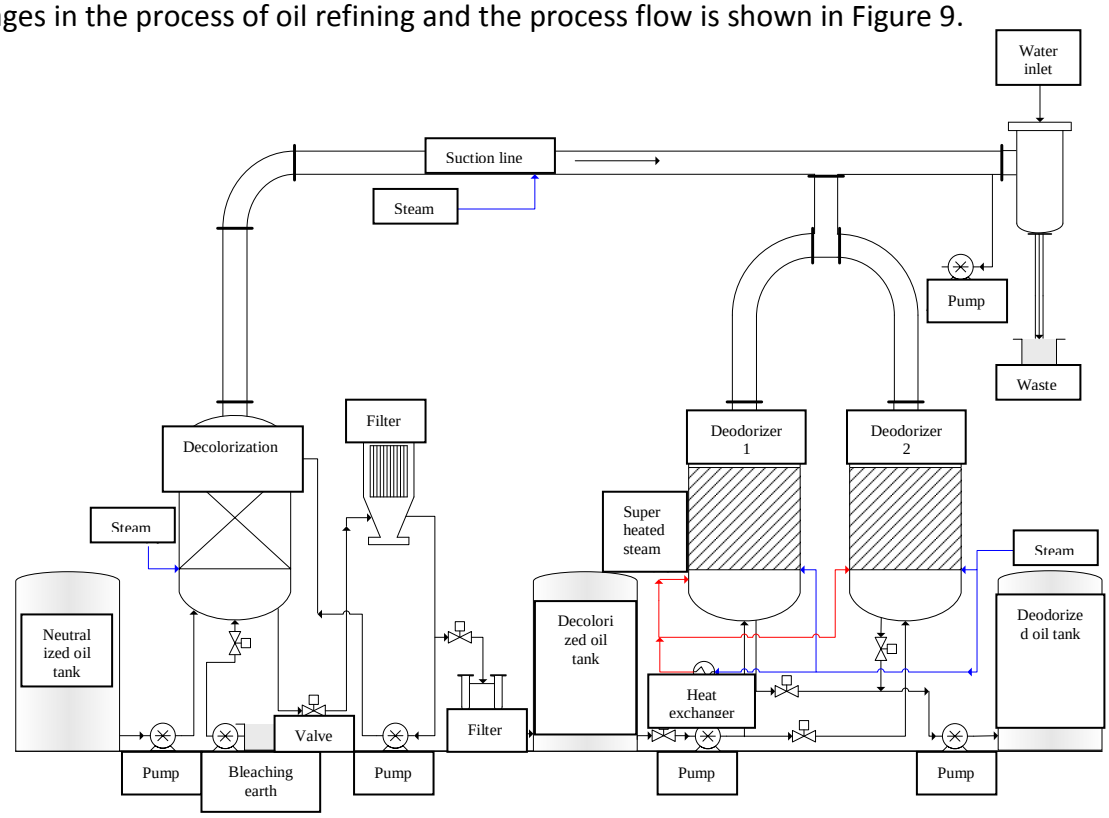


Figure 9. Bleaching and deodorization: Components and process flow

In the process of decolorizing the oil, the latter is placed in a container under vacuum, and heated by means of steam passing through pipes located within the container. After the temperature of the oil reaches 120 °C, bleaching earth is added and the mixture is being stirred at the same time. The mixture remaining in the vessel reaches discoloration usually in 20 minutes while the amount of added bleaching earth depends on the quality of the oil to be treated. Then the mixture is passed through a special filter (figure 11) so as to separate the oil from the bleaching earth. After separation, the oil is passed from a last filter, cooled and ready for either storage or for subsequent processing.

Various substances are removed from the oil after bleaching process, affecting its color. Also to be removed are substances that affect the quality and life of the oil such as phospholipids, soaps and metals, among others.

The main components of the unsaponifiable part of all types of oil are hydrocarbons reaching up to 10-30%. Among them dominates beta-carotene and squalene, which is the main component of unsaponifiable fraction (34 wt%) .

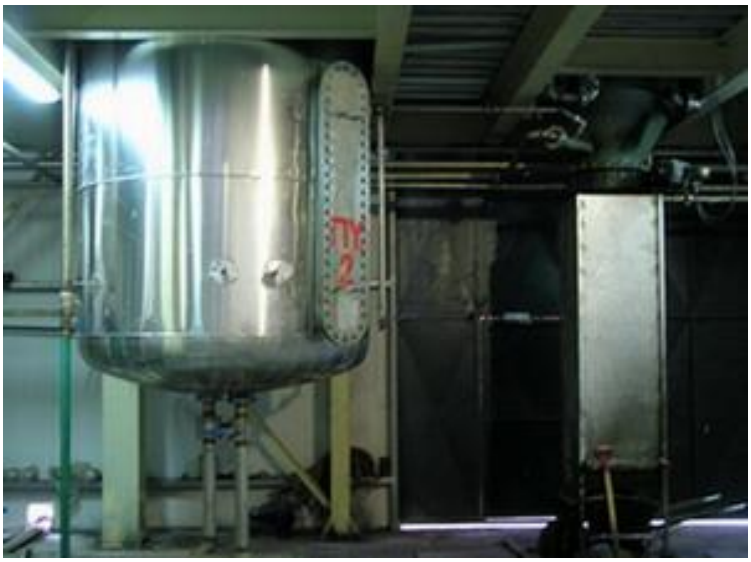


Figure 10: Oil bleaching container

Figure 11. Oil separation filter and bleaching earth

After the oil decolorization, this is transferred in two containers where the deodorization process will take place. Inside these containers there is a piping circuit for heating and cooling of the oil, depending on whether steam or water passes through. Initially from the circuit is passing steam and the oil is heated inside the containers up to 200°C under vacuum. Simultaneously superheated steam is blown from the bottom of the two containers for the stirring of the oil. The oil remains in the containers under vacuum for about eight hours and odors and unwanted oxidizing volatiles are removed via suction line (Figure 13). The suction line leads the volatile components, with the aid of a vacuum pump, to a device which condenses them with the help of water (Figure 14). Thus the liquid waste collected in special tanks. After the deodorization process the refined oil is collected in reservoirs for release onto the market.

Figure 12. Tanks for the storage of deodorized oil

Figure 13. Suction line

Figure 14. Mechanism of liquefaction of volatile waste

1.8.2) Olive (kernel) oil refining by-products: A source of valuable bioactive compounds.

The content and amount of by-products of the refining are affected by extraction technology and type of refining process (chemical versus physical refining) in addition to

the parameters affecting the quality of the oil such as origin, cultivar, state of ripening of the fruit, climatic conditions, and rainfall. One of the most important by-products of the refining is the deodorizer distillate. This residue is a very complex mixture containing free fatty acids (FFAs), phytosterols, tocopherols, sterol esters, hydrocarbons and breakdown products of fatty acids, aldehydes, ketones, and acylglycerols. However, the choice of column and operating conditions during the refining process determines the composition of deodorizer distillate. In olive oil deodorizer distillate (OODD), undoubtedly, squalene is the most important component due to antioxidant properties [Dumont et al., 2007]. Several methodologies have been developed for the efficient recovery and isolation of squalene from this by-product, comprising supercritical fluid countercurrent fractionation [Bondioli et al., 1993, Fornari et al., 2008, Akgun, 2011]

1.9) Table olive debittering process

Various types of procedures exist for the processing of table olives depending on the variety. In the following chapters, the two most applied processes are described.

1.9.1) Manufacturing process for Spanish-type olives

The most common method for the production of table olives (especially green) is the so-called Spanish method. The following figure shows diagrammatically the commercial production of this type:

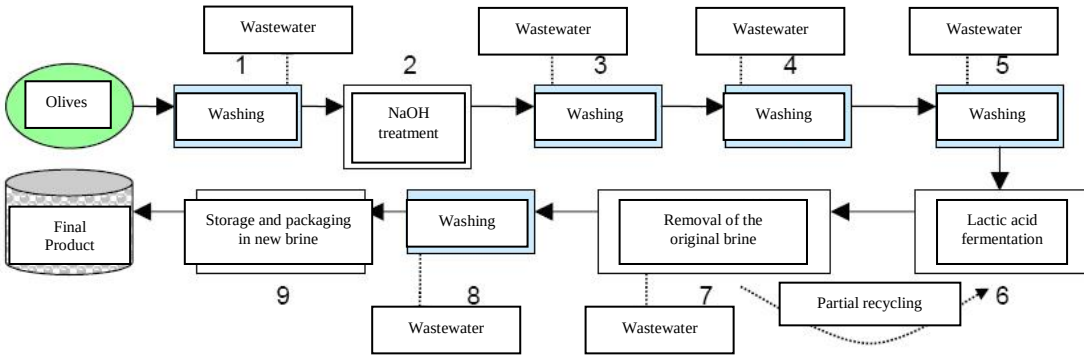


Figure 13. Process flowchart for the Spanish-type table olive production

Of a total of nine stages of processing the fruit, the five relate to washing and creating wastes. Three washings are necessary after treatment with sodium hydroxide (6-10 hours),

so as to remove the excess alkali from the flesh of the fruit. Sodium hydroxide hydrolyses oleuropein, which is responsible for the bitter taste of the fruits) into two components hydroxytyrosol and the glycoside of elenolic acid. Caustic soda debitters olives and fermentation ensures the diffusion of hydroxytyrosol and elenolic acid from the fruit flesh in the liquid fermentation medium (brine) [Soler et al. 2000, Salvador et al. 2001].

Fermentation is similar to that of other vegetables (e.g. cucumber) and due to lactic acid bacteria (Lactobacillus plantarum and Lactobacillus pentosus). The total duration of the fermentation ranges from 100-200 days [Salvador et al. 2001].

The green olive is collected in the early stages of ripening, but after the fruit has reached its normal size. The blonde olives are harvested before they are fully riped (semi - ripe fruit) and during the color transition from green olive to black , giving blonde–red color. Finally, the black and Kalamata olive are collected in full natural maturation of the fruit, and present a dark coloring. Subsequently the olives are transferred to the processing plant, wher the sorting takes place. The process, i.e. the stages of natural debittering and natural lactic fermentation of the olives, consists of placing the fruit firstly in aqueous sodium chloride solution (salt water, brine) acidified with a small amount of lactic acid. Salt is then added gradually, so that at the end of natural fermentation, salinity is 7.5 % minimum. Furthermore, lactic acid may be added to adjust the pH of the brine.

The time required for the natural fermentation of the fruit depends on the ambient temperature, the variety of olive and microbial population. After completion of fermentation, when exhausted those compounds used by fermenting microorganisms, olives kept in the same initial brine until its packaging. The fermentation of the olives is performed in most cases with the natural flora present in the drupe.

The major problem associated with the production of table olives is the management of wastes of the processing. These are abundantly produced as it can be seen in Figure 12. The main actor in the environmental impact is NaOH used in the first stage of the Spanish- type olive debittering, the NaCl used in almost all types of olives for the fermentation process and particularly the phenolic compounds extracted in the brine during the fermentation process. Especially phenols are a major problem because if not removed they hamper the waste management through biological cleaning. Although many methods of treating waste have been tested so far, these have failed to recover polyphenolic components as well as other interesting components such as triterpene naturally contained in the waste. All

existing methods of treatment aim at the destruction of these substances having as a consequence the loss of an important source of bioactive substances.

In Table 4, the applied debittering process is described, according to the variety.

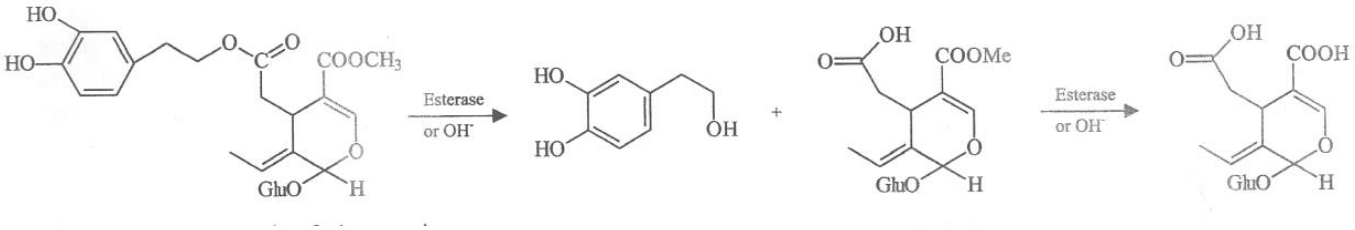
Olive variety	Procedure
1.Black olives	
Naturally ripe black olives in brine (Greek type)	Fermentation in 6.8% brine under anaerobic conditions for 3-6 months (The attenuation of the color corrected by aeration or Fe2+). The bitterness is removed partly in the brine. The olives have a fruity flavor and usually maintain a slightly bitter taste. Packaging in brine, sterilization or pasteurization or addition of olive oil and vinegar .
Naturally ripe black olives (type of Kalamata)	Debittering in water or brine. Fermentation in brine during 5-8 months. Packaging in brine, sterilized or pasteurized , or addition of olive oil and vinegar .
Naturally ripe black olives/drying by heating	Drying in the sun, until the bitterness is acceptable or drying in oven at low temperature (50C), for a few days to remove the bitter taste .
Processed black olives	Short treatment with NaOH (few hours) and then fermented in brine. Maintenance on new brine, sterilization or pasteurization and adding preservative.
Unprocessed naturally black olives/dry salting (Throuba type)	Ripe (or slightly wrinkled) black olives. Bloating with dry salt and undergo drying instead of fermentation. Then placed in alternating layers of dry salt in baskets, at room temperature for 20 to 60 days, to be dehydrated and debittered, thus they look wrinkled . Taste is a bit salty and bitter.
2. Olives artificially blackened	
Unprocessed olives artificially darkened in brine	Ripe green olives. Natural fermentation in brine 8-10% for 6-12 months.
Processed olives artificially blackened	Treatment with NaOH. Maintenance by natural fermentation or heat treatment.
Artificially blackened California Press (darkened by oxidation)	Ripe green olives. Storage in brine 5-9%. The processing is performed with 1.2 % NaOH. The alkaline solution is removed and there are intermediate rinses with water twice daily for 3-4 days with simultaneous aeration to obtain black , until pH 8. Add iron salts, for colour improving. Packaging in brine and sterilization.
3. Green - ripe olives	
Unprocessed green olives in brine	Ripe green olives. Fermentation in brine 8-10% for 6-12 months
Spanish-style green olives	Unripe green to lemon yellow olive. Treatment with NaOH 1-2% for 8-12h, rinsing with water and fermentation (lactic acid) in saline 9.5 % , which can be completed in 2-3 months. The pH is usually 3.8 to 4.4 when the fermentation is completed. Maintenance in particular pH, packaging in glass jars with various concentrations of brine and sterilization or pasteurization, preservation, refrigeration or inert gas, without brine.

Table 4. Different types of olives and relevant table olive processing

1.9.2) Effect of processing method in olive polyphenols

1.9.2a) Spanish-type process:

In this method, the alkali hydrolyzes rapidly oleuropein, which is converted to hydroxytyrosol and oleoside-11-methylester. If the pH and the ratio of reactants are suitable, further hydrolysis of the carbomethoxyl group of the ester, yields 6-dicarboxylic-oleoside. The free acids are converted into salts. The glycoside of hydroxytyrosol is not hydrolyzed , the verbascoside is hydrolyzed to caffeic acid and hydroxytyrosol-1-O-rhamnosyloglycoside while other esters and glycosides are hydrolyzed completely.



Alkaline hydrolysis of oleuropein to hydroxytyrosol

When the olives are stored in brine, the diffusion of ingredients and hydroxytyrosol is being continued, along with the enzymatic hydrolysis with the main product being hydroxytyrosol so that practically the concentration of the latter in the flesh remains stable.

In Greek varieties Chalkidiki and Konservolia, produced with Spanish-type method, mainly hydroxytyrosol, tyrosol and luteolin were detected in the final product and total phenols, expressed as caffeic acid, ranged from 354 to 1222mg/Kg.

1.9.2b) Greek-type process: Naturally black olives in brine

During the long period of fermentation in brine (> 6 months), in the flesh takes place at a slow rate the enzymatic hydrolysis of oleuropein and the polymerization of anthocyanins leading to color stabilization. In this type of olives usually yeasts dominate against lactobacilli which are inhibited by oleuropein.

In the first days of fermentation mainly hydroxytyrosol-4b-glucoside and oleuropein as well saligroside and verbascoside, have been detected which are then transformed into

hydroxytyrosol and Tyrosol [Romero et al. 2004]. After 12 months, the main phenol is hydroxytyrosol .

In the greek variety Konservolia elaborated by the above process, finished products (table olives) contain mainly hydroxytyrosol, tyrosol and luteolin [Blekas et al. 2002].

1.9.2c) Unprocessed natural black olives: dry salting (Throumba type)

In the ripe fruit variety throumba of Thassos, before processing, in the flesh there have been found (aqueous extract): oleuropein 1,81 mg/g, dimethyloleuropein 0,22 mg/g, hydroxytyrosol 0,28 mg/g, tyrosol 0,11 mg/g, hydroxytyrosol glycosides (hydroxytyrosol-1-O-gly, 3-O-gly and 4-O-gly: 0,23, 0,36, 0,80 mg/g, respectively), tyrosol-glycoside (0,23 mg/g), cornoside (1,34 mg/g) and halleridone (0,17 mg/g) [Boskou et al. 2006]. A recent study of the laboratory of pharmacognosy of UoA showed that throumba Thassos olives are an important source of oleuropein which remains in large quantities in the flesh, without being hydrolyzed because of the production process with dry salt [Zoidou et al. 2010].

1.9.2d) Table olive processing wastewater: A natural source of polyphenols

A typical composition of wastewater during processing of table olives is described in the table below.

Characteristic	**NaOH & Washing water**	**Brine**
pH	9 -13	4
NaOH [g/L]	1,1-1,5	-
NaCl [g/L]	-	6-10
Free acidity [g lactic acid/L]	-	6-15
Polyphenols [g tannic acid/L]	4,1-6,3	5-7
COD [g O2/L]	23-28	10-20
BOD5[g O2/L]	15-25	9-15
Soluble organic solids [g/L]	30-40	10-20

Table 5. A typical composition of wastewater during processing of table olives

However, the composition can vary depending on the olive variety, method of harvest etc. For processing table olives, 1 kg requires approximately 1.2 liters of water. The annual production of table olives in EU results over 750,000 tons of waste to be processed before being disposed.

The generated wastewater of table-olive processing contains a high amount of polyphenols which are endowed with interesting biological activities. These polyphenols are mainly hydroxytyrosol and tyrosol [De Castro et al., 2001, Bouaziz et al. 2008]. Additionally, at the end of this procedure the wastewater is usually discharged untreated to streams, creeks or directly to the sea. In other cases, it is transported to evaporation ponds, where malodors are a common nuisance, while the risk of polluting surface or ground waters is not always ruled out [De Castro et al., 2001, Brenes et al., 1995]. Valorization of by-products of industrial olive processing through the recovery of high added value compounds, such as polyphenols, represents a promising approach for diminishing the environmental impact of wastes and repositioning olive industry in highly competitive levels.

2) Innovation in natural products: Green extraction, enrichment and purification

2.1) Conventional vs. green processes

Extraction of bioactive substances from plants is not new: the food, cosmetic and pharmaceutical industry has been extracting bioactive compounds from plants for 30-40 years. Nevertheless a need has emerged for modernization of current techniques used at industrial scale such as solvent extraction to make them more economic and environment friendly. The solvents used are toxic, expensive, have low selectivity, need to be recovered by an energy-intensive operation, lead to thermal degradation and cause loss of valuable compounds during the various refining steps. For instance, extraction of carotenoids from vegetal sources such as tomato skins is also usually carried out using organic solvents, e.g., n-hexane, acetone, chloroform, ethanol, etc. These hazardous solvents are toxic, need specific disposal, are difficult to remove from final product (presence of solvent traces) and need a high temperature to work optimally, which means high energy requirements. To use another example, industrial processes commonly adopted to produce edible oils comprehend several stages, among them the extraction with n-hexane. However, this organic solvent is usually nonselective and accomplishes the simultaneous removal of non-volatile pigments and waxes, giving rise to dark colored and viscous extracts (oils) contaminated with solvent residues. This renders them difficult to handle without further

refining. The introduction of organic solvents threats the organic status of oils obtained from seeds of organic agriculture [Chemat et al., 2012].

2.2) Compressed fluids for the extraction of bioactive substances

On the other hand, compressed (or generally near-critical) fluids generally demonstrate good capabilities in natural products extraction. When referring to compressed fluids, we are generally referring to the following: Supercritical CO_2 (SC- CO_2 or SFE generally), Subcritical or Pressurized Hot Water (SWE or PHWE) and Pressurized Liquid Extraction (PLE) using mainly EtOH and/or ethyl lactate in order to maintain the advantage of using a green technology. They fulfill the basic goals of:

- reducing the amounts of solvents used for a certain quantity of plant material, through recycling
- simultaneously (in a single process) extracting multiple natural compounds
- increasing automation and throughput determination
- reducing time and overall energy required for the process, in comparison with conventional techniques such as maceration, percolation, soxhlet extraction etc.

As can be seen in Fig. 14, depending on the polarity of the compressed fluid selected, different ‘‘green’’ pressurized sample-preparation techniques can be used. All of them rely on the use of minimum amount of the foodgrade solvents for a selective extraction of bioactives while preserving their bioactivity and chemical structure. They all show great versatility and efficiency, since the physicochemical properties of solvents (density, diffusivity, viscosity, and dielectric constant) can be modified by changing the pressure and/or the temperature of the extracting fluid, which also modify their selectivity and solvating power.

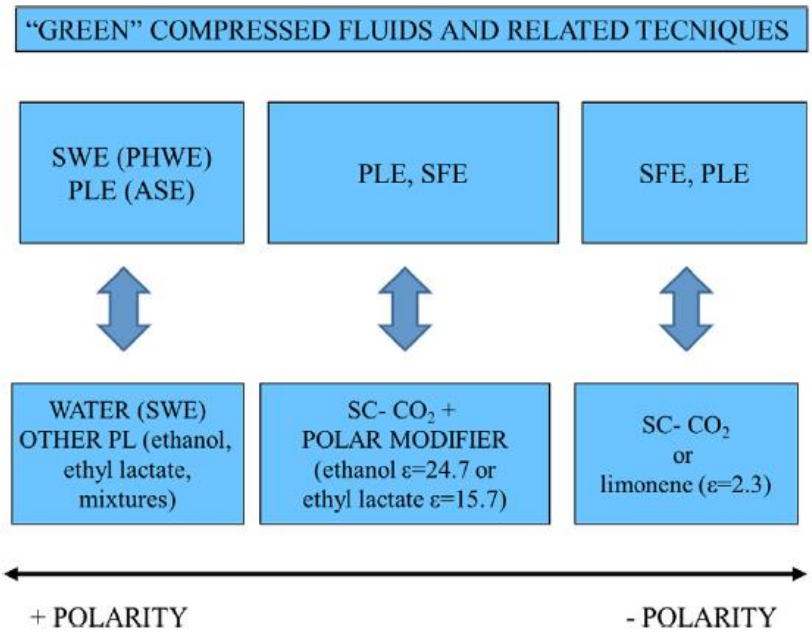


Figure 14. Green techniques based on the use of compressed fluids for extraction of natural products, ε=dielectric constant

2.2.1) Supercritical fluid Extraction

Supercritical fluids extraction (SFE) -and more specifically supercritical CO_2 extraction- is a suitable alternative to the conventional extraction techniques, since this solvent allows working at moderate temperatures avoiding thus thermal degradation, it is non-toxic and easily separated from the extract as it decompresses in atmospherical conditions during sample collection. The use of supercritical fluids as an alternative solvent for extraction has been attracting widespread interest owing to their particular properties (e.g., liquid-like solvent power, negligible surface tension, and gas-like transport properties), and changes in environmental regulations which foster the utilization of green solvents. In this field, CO_2 has been especially adopted since it is non-toxic, non-flammable, and inexpensive at industrial level; it can be recycled, has easily accessible supercritical conditions and is totally dissipated from extracts at atmospheric pressure avoiding the necessity of expensive and harmful refining treatments. Thus, CO_2 is the solvent most commonly used to extract bioactive compounds from natural sources using SFE. Despite some other solvents having been proposed for SFE (namely, e.g., propane, butane, and dimethyl ether), none of them fulfill the principles of GAC as well as CO_2. CO_2 has interesting properties for natural products extraction:

- its critical conditions are easily attainable (31.1°C and 71.39 MPa)
- it is a non-toxic, non-flammable solvent
- it is Generally Recognized as Safe (GRAS) for use in the food industry.

At supercritical conditions, solvents present high diffusivity, whereas their solvent strength and density can be easily modified by tuning the temperature and the pressure applied. Another important characteristic of this technique, when using supercritical CO_2 ($scCO_2$), is the possibility of attaining solvent-free extracts. Once the extraction procedure is finished, depressurization of the system turns CO_2 to gas, while the compounds extracted from the matrix precipitate. However, an important drawback of CO_2 is its low polarity, which can be overcome by employing low amounts (1–10%) of polar modifiers (EtOH, MeOH etc.) to change the polarity of the supercritical fluid and to increase its solvating power towards the analyte of interest.

2.2.1.1) Parameters affecting SFE

The most significant parameters that seem to affect the extraction performance significantly are the following:

- The pressure of the supercritical fluid is a crucial parameter affecting its being in a supercritical state or not. More specifically it affects immediately the solvents' density, and thus viscosity and diffusivity into the plant material. Additionally, pressure variations cause variations in the dielectric constant (ε) of CO_2, a measure of its polarity. Augmenting the pressure, results in an augmentation of the dielectric constant. The higher the dielectric constant of a solvent, the more polar this is. As it is known though, supercritical CO2 is a solvent of very low polarity. It exhibits a dielectric constant of ≈1.5 in the liquid state; supercritical CO2 will exhibit values generally between 1.1 and 1.5, depending upon density. This low dielectric constant can be both a process disadvantage and a chemistry disadvantage. Some reactions, for example, require polar solvents for best results. Further, low dielectric constant also suggests poor solvent power, and hence solubility in CO2 can require much higher pressures for certain classes of solute than more polar compressible fluids [Beckman, 2004].

- The temperature of the process is a crucial parameter that can affect the performance of the process, mainly in two different ways. First, by defining the state of the CO_2, being in supercritical conditions or not and affecting the solvents' density and thus viscosity and diffusivity into the plant material. Second, by modifying the extraction temperature, affecting thus the solubility as well as the chemical and structural stability of the various classes of compounds to the solvent or solvent mixture.
- Moisture content of the vegetable mass is an important parameter for SFE. That is because water, although it presents a very limited solubility in sc CO_2, can act as a co-solvent modifying the polarity and solvent capacity of the mixture. Indeed, increased moisture content of the vegetable material can lead to a limited solubilization of water into sc CO_2. This solubility is close to 0,8 mol/lt for a pressure of 250 bar and temperature of 40°C [Tassaing et al., 2004]. It is thus, capable of increasing quite significantly the sovents' polarity and thus the extracts' quality.
- Solvent to plant mass ratio is a parameter, along with the flow rate, that defines the duration of the extraction. That is because most often, sc CO_2 extraction in taking place in a dynamic mode. Its optimal values depend on mass transfer rate, or the rate of diffusion of the solutes from the plant structures to the solvent. Up to a certain point, the extract yield increases. Then it reaches a plateau where, under the current specific conditions, no solutes are further transferred to the solvent. That is when the extraction must be stopped or continued varying the experimental conditions, which will nevertheless most probably affect the extract quality as well.
- Proportion and type of modifier are key factors responsible for solubility of the target compounds in the supercritical fluid. Most often, a polar modifier is required in order to enhance polarity, solvent capacity as well as selectivity for various solutes, sometimes including relatively non-polar solutes [Dobbs et al, 1987]. Thus, such a co-solvent (an aliphatic alcohol usually) can facilitate the selective separation of solutes having different polarities, hydrogen bonding, and abilities for association and complexation. The organic modifier most commonly employed to extract bioactives is ethanol in a range 3–10% of CO_2 flow (m/m); other modifiers (e.g., methanol, acetone or even small amounts of water) have been also used to isolate

polar bioactive compounds, such as oleuropein, a glycoside from olive tree leaves [Xynos et al., 2011]. Medium to low polarity compounds such as sterols still present a relatively low solubility in pure supercritical CO_2, and need the addition of a polar modifier that although it does not enhance selectivity, it enhances solubility [Wong and Johnston, 1986]. On the other hand, in order to extract high molecular-weight non-polar compounds, vegetable oils (e.g., olive oil) have demonstrated ability as co-solvents at a proportion of 10% [Temelli, 2009]. Nevertheless, even polar modifiers such as ethanol can enhance very significantly –by orders of magnitude- solubilities of oils, such as sunflower oil, in sc CO_2, at 300 bar and 42°C [Cocero and Calvo, 1996].

- Particle size and use of dispersing agents have parameters more related to the efficiency of the extraction procedure and can be modified to avoid clogging and to increase extraction rate. The particle size (or crushing degree) is a very significant factor in the mass-transfer rate, so it will have maximum influence on the extraction yield.

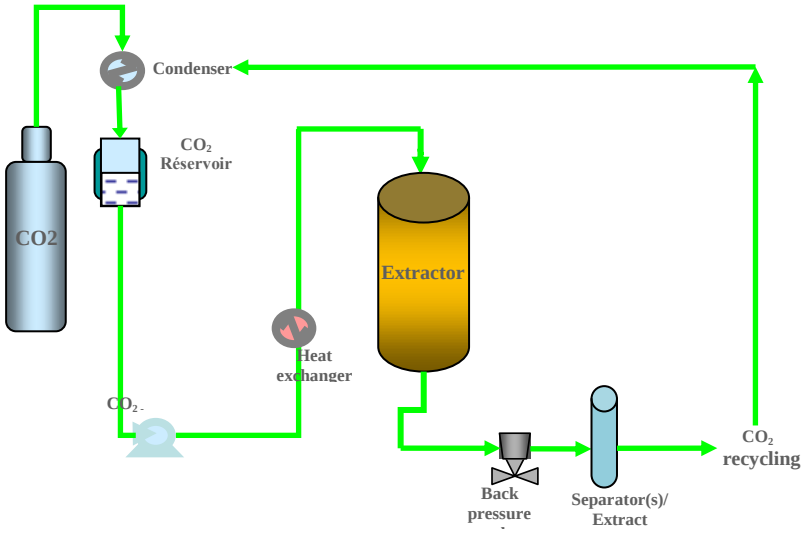


Figure 15. Process flowchart for a typical SFE apparatus

2.2.2) Subcritical water extraction

Subcritical water extraction (SWE) is a novel green technology for obtaining bioactive substances from plant material. The solvent in this case is superheated water (water in a temperature over its boiling point and raised pressure), which presents a lower dielectric

constant than water under 100ºC and thus, a modified polarity. More specifically subcritical water is less polar and it can solubilize compounds of medium to high polarity, such as phenolics in contrast with hot water that remains highly polar. For instance, quercetin has been isolated from onion skin by using SWE [Min-Jun Ko et al, 2011]. Thus, SWE is a particular type of PLE based on using hot water as the pressurized liquid. Usually, temperatures higher than the boiling point of water (100°C) and lower than its critical temperature (374°C) are used, while pressures of 3.5–20 MPa are employed to keep the water in the liquid state during the whole extraction process.

Under these conditions of pressure and temperature, physical and chemical properties of water change dramatically. Among the main parameters that can influence SWE (extraction temperature, time, pressure, the addition of an organic solvent or surfactant, and waterflow rate), temperature is the main factor that affects extraction efficiency and selectivity. An increase in temperature:

- facilitates analyte diffusion (diffusivity of water at 25°C is about 10 times lower than that of water at 200°C)
- favors mass-transfer kinetics by disrupting intermolecular forces (i.e. van der Waals forces, hydrogen bonds and dipole attractions)
- decreases the viscosity of water (enabling better penetration of matrix particles)
- decreases the surface tension (allowing the water to better wet the sample matrix) [Turner et al., 2011]

In spite of the improvement in all these properties, the most important effect of the increase of liquid water temperature is undoubtedly the weakening of hydrogen bonds, resulting in a lower dielectric constant (ε). The dielectric constant (measure of polarity) of water, at enough pressure to be maintained in its liquid phase, varies from 80 at 25°C (being extremely polar) to 25 – 27 when temperatures of 250°C are used [Ong et al., 2006], which falls between those of methanol (ε = 33) and ethanol (ε = 24) at 25°C [Ong et al., 2006 and Teo et al., 2010] (see Fig. 16). As can be observed, the dielectric constant values of water resemble those of other, less polar solvents at room temperature, so, under these conditions, water could be used as an alternative to dissolve medium-polar and even non-polar organic compounds.

Apparatuses for SWE are relatively new in the market and few constructors design and produce them. Nevertheless, SWE can be performed either using a PLE extraction apparatus [Cheigh et al., 2012], or by modifying SFE equipment (figure 17, "home-made" setups). The advantages of a home-made setup, compared to commercial systems, are: (1) the range of working temperature; (2) the possibility of carrying out both dynamic and static extractions; and, (3) different processes (extraction, reaction, drying) just modifying the basic set-up.

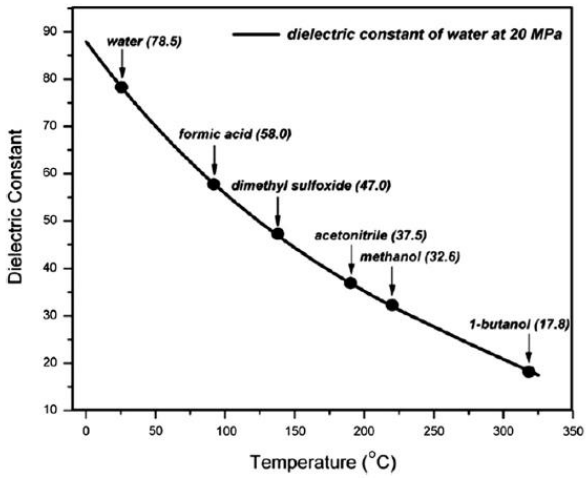


Figure 16. Dielectric constant of water as a function of temperature at 20 MPa. Spots on the plot are the values of dielectric constant corresponding to various organic solvents at 25°C and 0.1 MPa

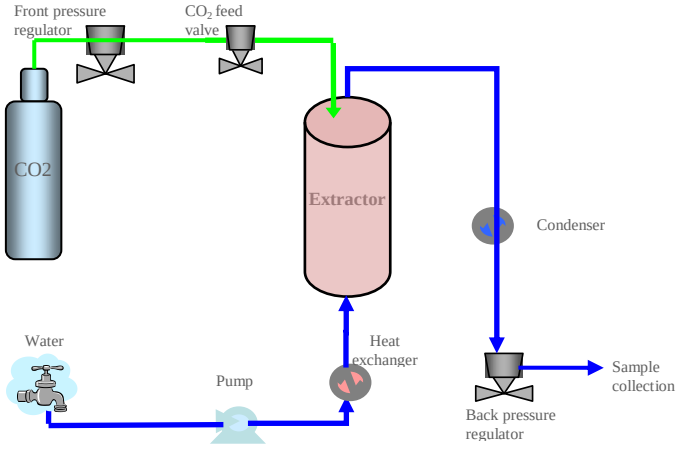


Figure 17. Process flowchart for a SWE apparatus

2.2.3) Pressurized Liquid Extraction

Pressurized liquid extraction (PLE) is a technique which uses classical solvents and performs a fully automated extraction under constant stable pressure and various controllable parameters like temperature, static extraction time, extraction cycles, duration of purging the extraction cell at the end of each extraction etc. The filtration of the final extract is

being performed automatically during its collection. Water and ethanol, as well as ethyl lactate can be efficiently used in PLE for the recovery of polar to medium polarity compounds, thus maintaining the environmentally-friendly properties. PLE is always taking place in specially designed apparatuses that provide a, most often, stable and non-modifiable pressure, usually around 100 bar (approx. 1500 psi). This, not only helps maintaining the solvent under pressure but also destroying the cellular structures of the vegetable matrix, deliberating thus more easily the desired solutes. Other extraction parameters, except of course from the solvent choice, can be varied in order to achieve the desirable results:

- The temperature of the process is a crucial parameter that can affect the performance of the process, mainly in two different ways. First, by defining the state of the solvent, being in near-critical conditions or not. This affects the solvents' density and thus viscosity and diffusivity into the plant material. Second, by modifying the extraction temperature, affecting thus the solubility as well as the stability of the various classes of compounds to the solvent or solvent mixture.
- Particle size is closely related to the efficiency of the extraction procedure and can be modified to avoid clogging and to increase extraction rate. The particle size (or crushing degree) is a very significant factor in the mass-transfer rate, so it will have maximum influence on the extraction yield.
- The static or dynamic nature of the process is a very significant choice that will define, along with the extraction cycles the saturation or not of the solvent in solutes and thus the extraction efficiency.
- Other parameters that participate in extraction programming, such as flushing, pre-heating, purging the solvent etc. during the extraction.

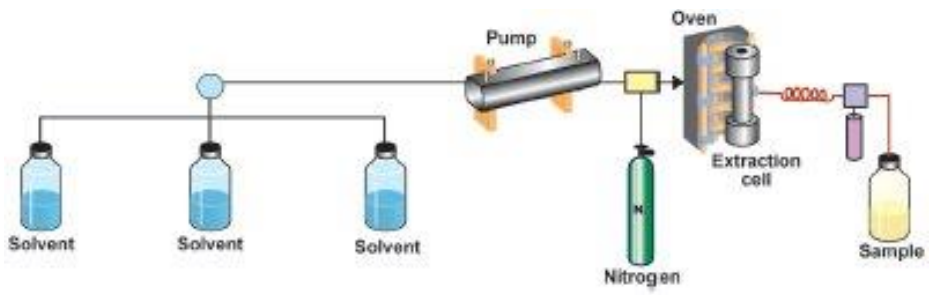


Figure 18. Process flowchart for a PLE apparatus

2.2.4) Adsorptive Resin Technology

Highly cross-linked adsorptive resins (AR) are characterized by the large number of permanent pores and have been developed for the purification of different categories of compounds. These include polyphenols, flavonoids, glycosides, carotenoids etc. Their functionality varies depending on its type. In polar solvents such as water, polymeric adsorbents exhibit non-polar behavior and can adsorb organic species that are sparingly soluble. In non-polar solvents they exhibit slightly polar properties. Adsorption by macroporous resins is considered to be superior- and more environmentally friendly- to conventional extractions due to low operational costs, lower solvent consumption and absence of chemical residues in the final product [Francisco et al., 1992]. Research as well as industrial application includes different fields such as nutrition, waste-water treatment, natural products etc [Young Ku et al., 2000]. Thus, AR technology can be implemented for the retention of relatively small molecules out of complex liquid matrices, reducing also the organic load of the latter.

The adsorbents that AR technology very often utilizes are of polymeric nature. Polymeric adsorbents can be thought of as highly porous structures whose internal surfaces can adsorb and then desorb a wide variety of different compounds, depending on the environment on which they are used. Often, in polar solvents, such as water, polymeric adsorbents exhibit non-polar or hydrophobic behavior and so can adsorb organinc species that are sparingly soluble. This hydrophobicity is mostly pronounced with the styrenic adsorbents (dipole moment= 0.4 debye). In polar solvents, such as hydrocarbons etc. most adsorbents exhibit slightly polar or hydrophilic properties and so will adsorb species with some degree of polarity. This polarity is mostly pronounced with the acrylic adsorbents (dipole moment=1.83 debye) and the phenolic adsorbents (dipole moment=1.63 debye). The adsorption of a particular compound also depends on its similarity to a particular adsorbent on the basis of “like attracts like” – hence acrylic and phenolic adsorbents have important applications also in aqueous media. In many cases it is the product of interest that is adsorbed onto the resin and thus its elution is very important and is considered a separate section. The most important parameters that have to be taken in consideration for the choice of the appropriate adsorbent for a certain application are:

- Nature of the solvent: This is related to the bulk properties of the solvent itself. The two main categories and polar and non-polar solvents.
- Functionality of the solute: Aromaticity, high local electron density, oxidatation and saturation are the main characteristics to be evaluated.
- Polarization: This can be related to the dipole moment or the dielectric constant of the solute. Also, capability of hydrogen bonding is crucial.
- Size of the solute: Small molecules are defined to have a MW<1.000 D whilst large molecules have a MW>10.000 D.

The interaction between the solute and the polymer is relatively weak when comparing to pure ionic or covalent bonding. In some cases, the attractive forces can be disrupted simply by raising the temperature and there are examples where even hot water at 60°C can be used to either elute or regenerate polymeric adsorbents. However, many of the applications of the polymeric adsorbents involve the isolation of thermolabile conpounds. Thus, other methods of weakening the solute-polymer interaction have been developed and these can be divided into two main categories, depending on whether the solute itself or the environment is being modified. In the first case, if the solute contains either a weakly ionic or a polarizable functionality, then this can be used, by modifying the pH of the relevant step, to either adsorb and then desorb the product. In the second case, a change in the solvent is necessary in order to achieve desorption. The new solvent has to have higher affinity for the solute than the medium to which the adsorption has taken place. Often, a solvent/aqueous mixture is sufficient to achieve desorption of the desired solutes.
A material that is being used extensively for phenol adsorption (i.e. decontamination of phenols from aqueous solutions, phenol enrichment of aqueous extracts etc.) is highly cross-linked, macroreticular polystyrene (AmberliteTM XAD4). This type of material, as well as other types –depending on the matrix and solutes chemical nature- is implemented in various procedures, mainly:

- Recovery of high value materials
- Purification
- Decolorization
- Capture of pharmaceuticals and biopharmaceuticals

- Detergent removal

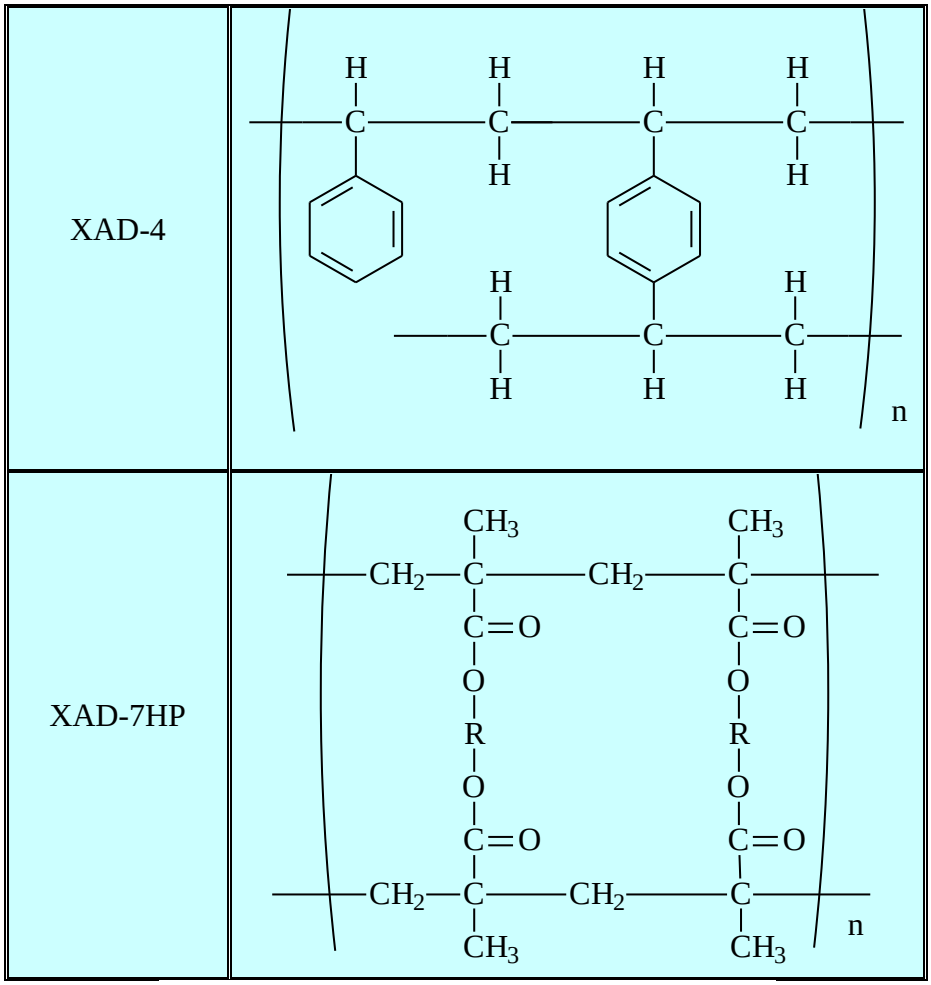


Figure 19. Chemical structure of XAD-4 and XAD-7 resins.

2.2.5) Liquid/liquid centrifugal extraction/separation

When operating as a centrifugal extractor for performing liquid-liquid extractions, a feed solution, containing one or more solutes (shown in yellow in Figure 20), and an immiscible solvent (shown in blue in Figure 20) with a different density than that of the feed solution are fed to the mixing chamber located on the bottom of the centrifuge housing. A rotating agitator disc mixes the two immiscible liquids into a dispersion (shown in green in Figure 20). Different agitator disc designs can be used depending upon the liquids' interfacial tension. The efficient mixing creates a large interfacial area between the two liquids to ensure maximum mass transfer of the solutes.

The dispersion is aspirated into the centrifuge bowl by a turbine located on the bottom of

the rotating bowl. The liquids are separated by the centrifugal force generated by the rotating bowl. The heavier liquid (shown in yellow on Figure 20) occupies the outer portion of the bowl. The light liquid (shown in blue on Figure 20) occupies the inner portion of the bowl. The position of the liquid / liquid interphase is regulated by a heavy phase weir. Interchangeable heavy phase weirs

of different diameters accommodate a wide range of density ratios. The heavy phase underflows to a static receiving chamber. The light phase overflows to a separate static receiving chamber. The liquids are discharged by gravity to the next BXP centrifugal extractor or to downstream equipment.

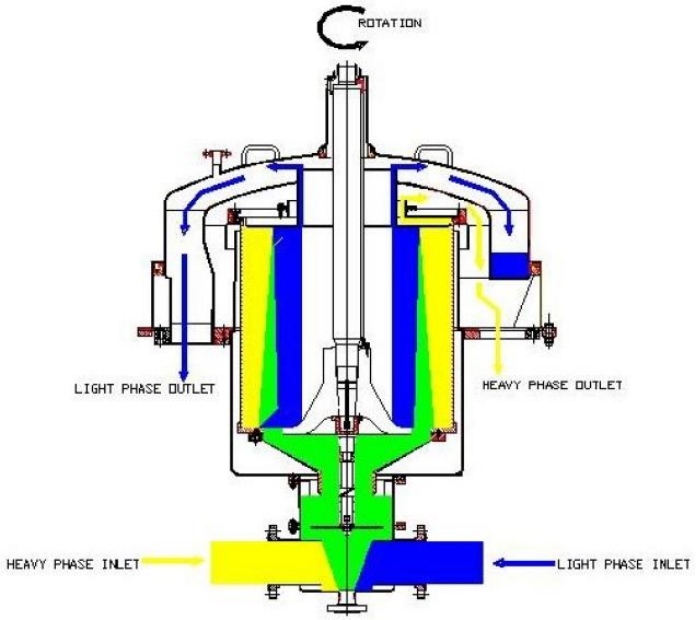


Figure 20. Single stage countercurrent centrifugal extractor

The performance parameters of a centrifugal extractor will vary depending on the solvents used, viscosity, temperature, density ratio, surface tension, and phase flow rate ratio. Specifically, for a centrifugal extractor, G-force and the mixing energy are important factors to consider during testing. Increased rotational speed provides a higher driving force for separation. However, as rotational speed increases, this will also increase the mixing energy imparted on the liquid / liquid

system. Therefore, the maximum rotational speed may not yield the best results. The vigorous mixing at the higher speed may create a dispersion that is more difficult to separate. Typically, there is a “bandwidth” of rotational speeds that balances the right

amount of mixing with adequate G-force for effective separation. RPM vs. G-force correlation diagram for centrifugal extractors can be seen in Figure 22.

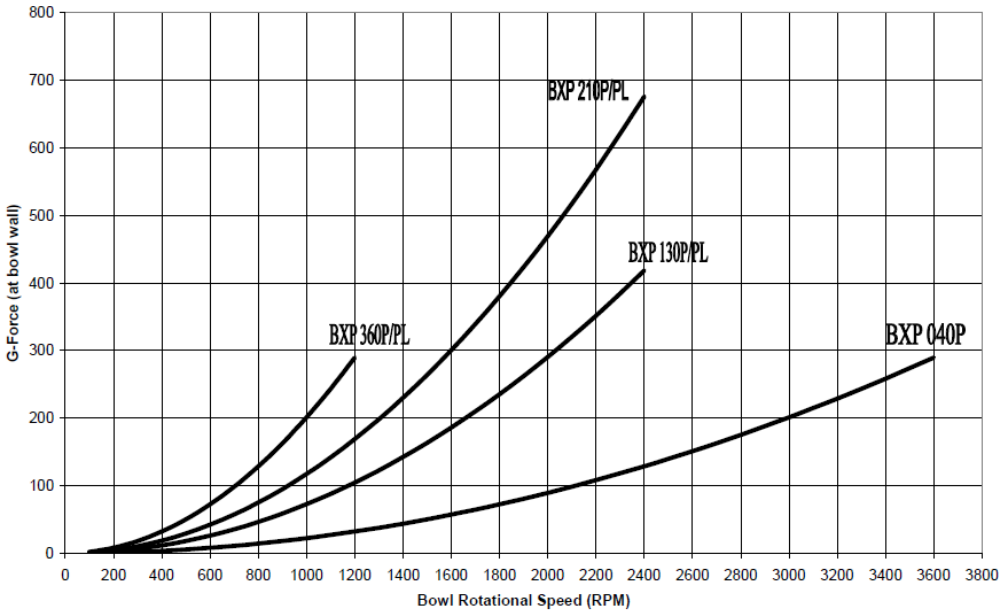


Figure 21: G-force vs. rotational speed diagram for monostage centrifugal extractor/separator.

2.2.6) Fast Centrifugal Partition Chromatography

Centrifugal Partition Chromatography (CPC) also alternatively called high-speed counter-current chromatography (CPC) is considered as the green chromatography, especially in preparative and large-scale. It is essentially a liquid-liquid partitioning technique that eliminates various complications arising from the use of solid supports. Separation takes places in the centrifuge using two immiscible liquid phases consisting typically of a mixture of multiple solvents in a specific ratio. The wide choice of CPC solvent systems means that the whole spectrum of polarities can be covered and compounds ranging from very hydrophilic to extremely lipophilic can be fractionated. Methods can be easily transferred to another apparatus, making the scale-up of separations and isolations feasible. CPC does not use any solid phase and thus it does not produce solid waste to be treated, while the

fact that usually one phase is aqueous reduces the use of organic solvents for the isolation of pure compounds.

More specifically, in high speed countercurrent chromatography a sample is partitioned between two non-miscible liquid phases. One phase is held stationary by a centrifugal force (applied by spinning the separation element at high speed), while the second phase is pumped through the apparatus. Unlike high performance liquid chromatography (HPLC), in which the stationary phase occupies 5-7% and the mobile phase about 75% of the column, the relative proportions in CPC are 50-75% for the stationary phase and 20-50% for the mobile phase. As a consequence, large sample loads are possible with CPC. Another important advantage of the absence of a solid support is that irreversible adsorption is avoided. There is total recovery of the injected sample and tailing is minimized. CPC is thus of special importance for the separation of sensitive and easily degraded samples. Although the efficiency of CPC separations is lower than that encountered in HPLC, the optimization of selectivity is the great advantage offered by the former technique. The potential of CPC is further shown by the possibility of applying gradients for separations. Solvent proportions can be changed during a chromatographic run. Furthermore, solvent elution can be reversed in the course of a separation by changing over stationary and mobile phases. Consequently, one of the characteristics of CPC is its extreme flexibility. While conventional liquid chromatography uses a single phase to elute the analytes released from the adsorptive or liquid phase coated solid support, the CPC technique uses a *two-phase solvent system* made of a pair of mutually immiscible solvents, one used as the *stationary phase* and the other as the *mobile phase*. The use of two-phase solvent systems allows one to choose solvents from an enormous number of possible combinations. The selection of this two-phase solvent system for the target compound(s) is the most important step in CPC where searching for a suitable two-phase solvent system may be estimated as 90% of the entire work in CPC. Several monographs, review articles, and book chapters in the cited references describe various two-phase solvent systems successfully used for CPC. Without consulting the literature, the search for a two-phase solvent system for the successful separation of particular compounds from a complex sample mixture can be very time-consuming. The selected solvent system should satisfy the following requirements [Ito, 2005]:

- the analyte(s) should be stable and soluble in the system;

- the solvent system should form two phases with an acceptable volume ratios to avoid wastage
- the solvent system should provide a suitable *partition coefficient* to the analytes; The *partition coefficient*(*K*) is the ratio of solute distributed between the mutually equilibrated two solvent phases. Usually it is expressed by the amount of solute in the stationary phase divided by that of the mobile phase as in conventional liquid chromatography. One must find systems with K values of the target compounds in a proper range: The suitable K values for CPC are $0.5 \leq K \leq 1.0$. A smaller K value elutes the solute closer to the solvent front with lower resolution while a larger K value tends to give better resolution but broader, more dilute peaks due to a longer elution time. Before deciding which phase is to be used as the stationary phase, the user therefore may temporarily express the partition coefficient as $K_U/L = C_U/C_L$, where C_U is the solute concentration in the upper phase and C_L, that of the lower phase. If $K_U/L = 2$, the lower phase should be used as the stationary phase, which gives $K = 0.5$.
- the solvent system should yield satisfactory retention of the stationary phase in the column; The CPC system uses no solid support to retain the liquid stationary phase in the separation column. In CPC, the retention of the stationary phase is accomplished by a combination of coiled column configuration and the planetary motion of the column holder. Successful separation in CPC largely depends on the amount of the stationary phase retained in the column. In general, the higher the retention of the stationary phase, the better the peak resolution. The amount of stationary phase retained in the column is highly correlated with the settling time of the two phases in a test tube, thus one must measure the settling time of the two-phase solvent system to be used for the separation, inside a test tube.

Most applications of CPC have been performed on hydrostatic equilibrium instruments. These have either cartridges or discs arranged around a central axis. The separation column, in effect, consists of a series of cells in the cartridges or discs. Practically, the whole apparatus is first filled with the stationary phase of a biphasic solvent system and the sample is injected. The mobile phase is then pumped via the sample chamber into the column, forming a stream of droplets in the immiscible stationary phase. Depending on the

choice of solvents for the mobile and stationary phases, these droplets are made either to ascend (“ascending mode”) or descend (“descending mode”) through the column. As the mobile phase moves through the column in the form of droplets, turbulence promotes efficient partitioning of the solute between the two phases. Separation occurs according to the difference in the partition coefficients of the components of the sample.

The separation of various classes of natural compounds has been described in bibliography extensively. This comprises secondary metabolites such as flavonoids, xanthones, chalcone derivatives, phenylpropanoids and coumarins, lignans, tannins, mono- and triterpenes and their glycosides, alkaloids, secoiridoids and more [Marston and Hostettmann, 2000].

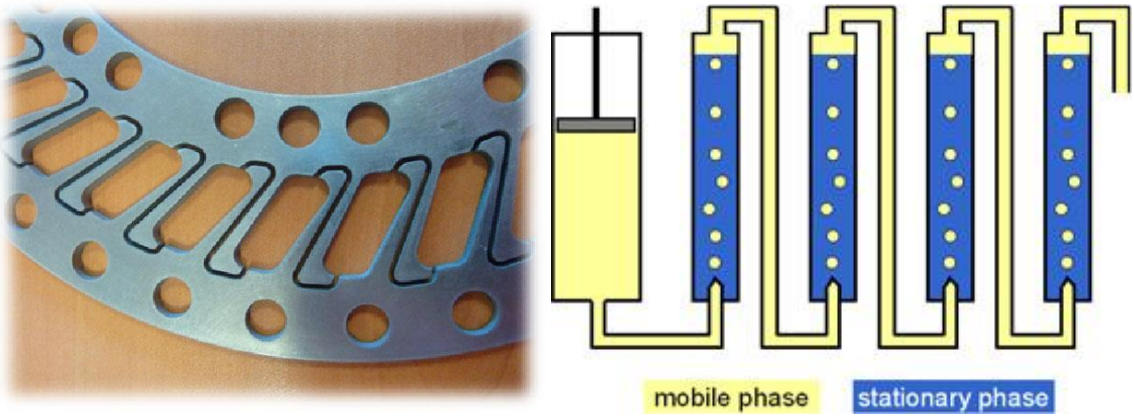


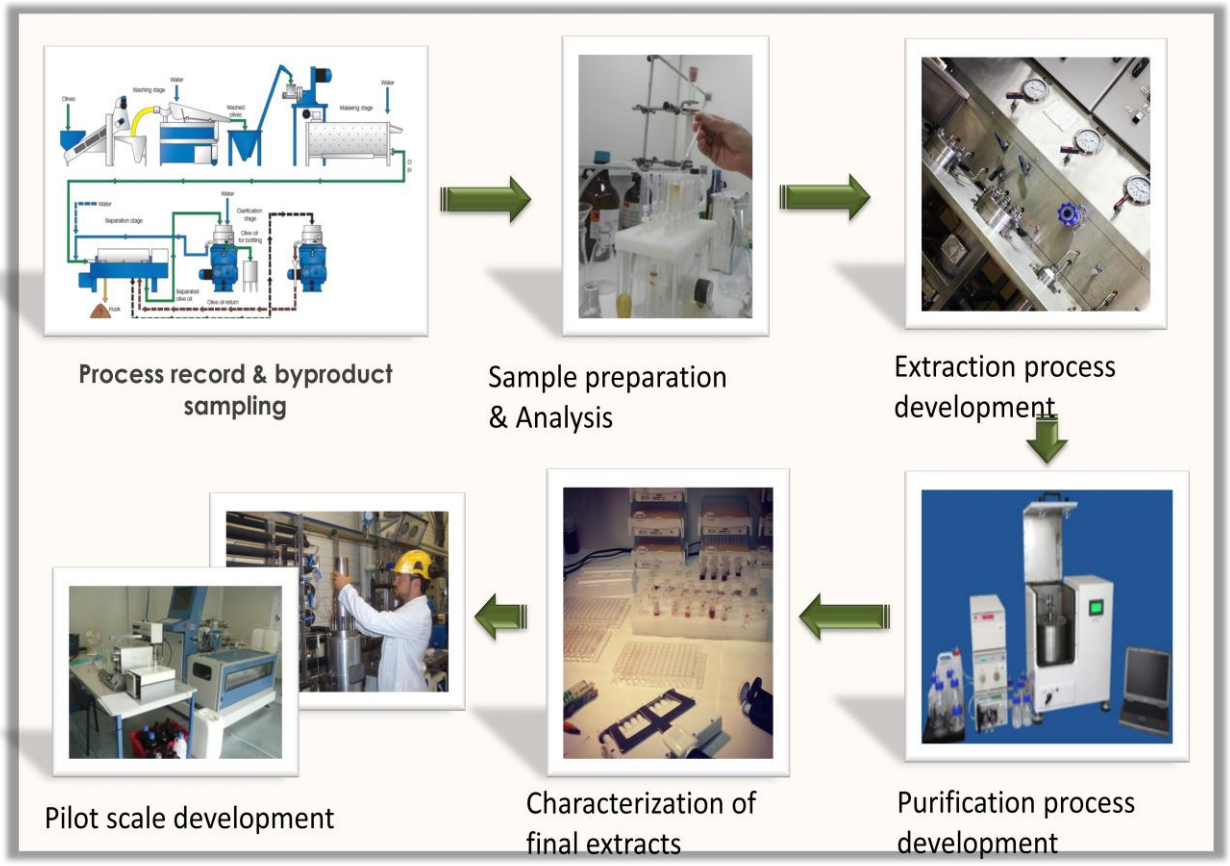
Figure 22. Design of successive separating funnels in a CPC column and working principle

PART B – EXPERIMENTAL PART

1) PhD experimental overview

1.1) Workflow axis

The axis of the workflow of this thesis is summarized as follows:



1.2) Experimental work

1.2.1) State-of-the-art environmentally friendly extraction technologies elaborating super/subcritical fluids for the production of high quality olive leaves' extracts.

1.2.1.1) Aim of the study

The aim of the present study was to establish a clean, fast, highly sensitive, automated method for isolation of bioactive compounds taking advantage of the selectivity of SC-CO2 for non-polar compounds and the use of polar solvents in their liquid or subcritical state at PLE. In this respect contemporary environmentally friendly technologies were assayed for

their potential in effective production of high yield extracts, rich in oleuropein, with high antioxidant capacity from olive leaf matrix, thus making easier and more accurate their subsequent application in the fields of medicine, cosmetics and food.

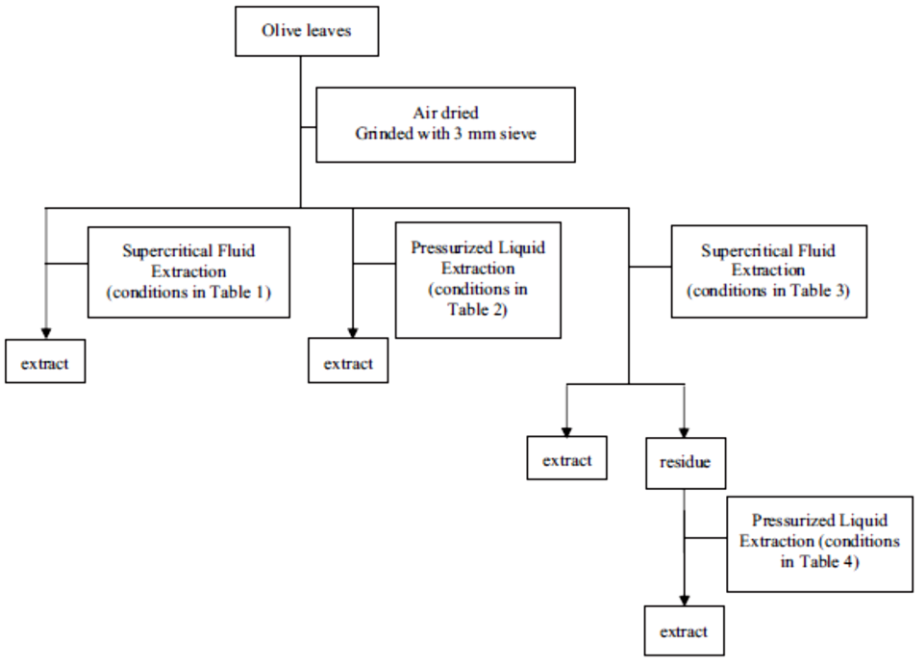


Figure 23. Schematic representation of experimental procedure

1.2.1.2) Materials and methods

1.2.1.2a) Solvents and reagents

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) reagent was obtained from Sigma–Aldrich (USA). Carbon dioxide (for the extraction procedure) was supplied by Air-Liquid Hellas in cylinders and the absolute ethanol (for DPPH tests and supercritical CO2 extraction apparatus) was obtained by Carlo Erba Reagents. Methanol (HPLC grade) was obtained from Merck KGaA, Darm-stadt and acetic acid 100% glacial from Riedel-de Haën. HPLC-grade water was obtained by double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, MSU).

1.2.1.2b) Plant material

Olea europaea (variety Koroneiki) leaves were collected in 2009 in the region of Attica, dried in a well ventilated shady place and subsequently stored. Before the extraction the leaves were grinded using an AllenWest type SCIS grinder with a sieve of 3 mm.

1.2.1.2c) Extraction apparatuses

The supercritical fluid extraction was performed in a pilot-scale apparatus (SFE 1-2 No. 4218, SEPAREX), which is designed to allow the study of a wide range of conditions. It consists of a CO2 tank, a liquid CO2 pump (that can deliver up to 10 kg/h), 2 extraction vessels (1 L and 2 L, respectively) which are both connected directly and parallel between them, 3 separators (with 200 mL capacity each), a co-solvent pump (with 40 mL/min maximum flow rate) and a cooling system. The extractions were performed within a stainless steel basket placed in a 1 L tubular extractor. The pressure in the separators compartment was kept constantly at 5 MPa. The extraction temperature was 50 ◦C and the separation temperature was 55 ◦C. The extraction was dynamic with continuous recycling of the sol-vent. For more details on the extraction parameters see tables in the results section. Finally, the extracts were evaporated to dryness using a rotary evaporator (Buchi Rotavapor R-200) at 45 ◦C and subsequently submitted to lyophilisation.

A Dionex Accelerated solvent extraction (ASE) 300 System (Dionex, Sunnyvale, CA) with 100 mL stainless steel vessels was used for the pressurized liquid extraction (PLE). Specifically 6.0 g of grinded olive leaves powder were placed each time into the tubular extraction cells. These were then placed into the carousel and the samples were extracted under the specified conditions. The pressure applied was kept constantly at 10.34 MPa. The procedure was static. For more details on the extraction parameters see tables in the results section. Finally, the extracts were evaporated to dryness using a rotary evaporator (Buchi Rotavapor R-200) at 45 ◦C and subsequently submitted to lyophilisation.

Figure 24. SFE apparatus (SFE 1-2, Separex) in University of Athens, Laboratory of Pharmacogosy

Figure 25. PLE apparatus (ASE 300, Dionex) in University of Athens, Laboratory of Pharmacogosy

1.2.1.2d) HPLC analysis

The quantitative determination of oleuropein was performed in an HPLC-DAD system: Thermo Finnigan HPLC system (Thermo Finnigan, San Jose, CA) coupled with a Spectral System UV6000LP PDA detector. A two solvent gradient method was used: A. H2O + 1% acetic acid and B. MeOH. The flow rate was set at 1 mL/min and the following elution program was applied: 0–2 min linear gradient to 5% B; 2–10 min linear gradient to 25% B; 10–20 min linear gradient to 40% B; 20–30 min linear gradient to 50% B; 30–34 min 50% B isocratic; 34–45 min linear gradient to 90% B; 45–50 min 90% B isocratic; 50–60 min linear gradient to 100% B; 60–65 min 100% B isocratic. Available standard solution of oleuropein was prepared in 50% aqueous/methanol and run under the same conditions as the

samples. The analysis was performed at 25 ◦C and the injection volume was 20 µL. The detection was done at 248 nm and the column used was Supelco Analytical Discovery HS C18 (25 cm × 4.6 mm i.d., 5.0 µm). 2.6. TLC analysis Merck silica gel 60 F254 (Art.5554). Detection: UV-light, spray reagent (vanillin–H2SO4 on silica gel). 2.7. Antioxidant activity – DPPH method DPPH (2,2-diphenyl-1-picrylhydrazyl) is a simple and inexpensive method to measure the antioxidant capacity of extracts. It is a free radical that has the ability to be reduced to 2,2-diphenyl- 1-picrylhydrazine in the presence of antioxidant compounds. The assay was based on the publication of Lee et al. [21]. The procedure involves dissolving 12.4 mg of DPPH in 100 mL of ethanol. Then 10 µL of the solution is added to 190 µL of an extract solution (dis-solved in DMSO) and the mixture is left for incubation (at 37 ◦C) for 30 min in the dark and the absorbance is measured at a wavelength of 517 nm. All the samples were tested in a final concentration of 200 µg/mL and active were considered the extracts that reduced the free radical at a percentage >80%. All the experiments were per-formed in triplicate. The whole assay took place in the 96 well plate using an Infinite m200 pro TECAN reader (Tecan Group, Männedorf Switzerland).

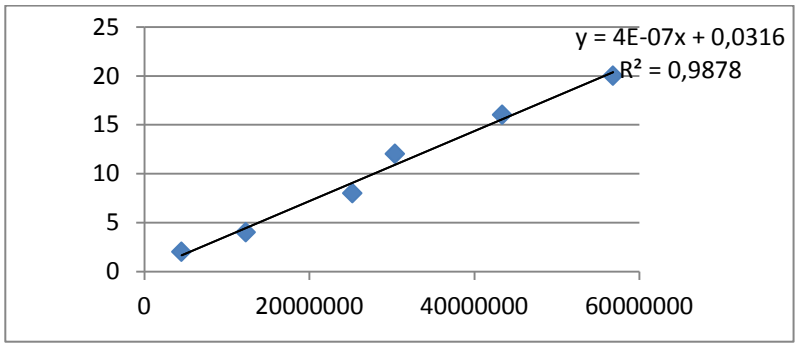


Figure 26. Calibration curve for Oleuropein in HPLC

1.2.1.3) Results and discussion

Dried and pulverized olive leaves were treated with methodologies developed on SFE and PLE having as a goal to obtain high yield extracts, rich in oleuropein that would exhibit high radical scavenging activity. State-of-the art super/subcritical fluid extractions with CO2, water and ethanol were used, exploiting the unique features of each technique (Fig. 1). As a first approach olive leaves (275.0 g) were extracted with SC-CO2 modified with ethanol in

order to investigate the amount of oleuropein that would be possible to recover and the required parameters, mainly concerning the necessary amount of co-solvent. The fact that CO2 is considered an apolar solvent rendered necessary the addition of ethanol as co-solvent. Ethanol was selected because of its low toxicity compared with other options. Thereby, the applied pressure was 30 MPa, in order to increase fluid density and solvating power of CO2 and the percentage of ethanol initially reached 5%. Under these conditions the yield of the dry extract was 14.7% and no oleuropein was detected. Following this, pressure remained at a high level and the percentage of ethanol was increased to 20%. These parameters had as a consequence the acquisition of 46.75 g of dry extract (17.0% yield) (Table 6). In addition, the extract was analyzed for oleuropein (Fig. 24). The dry extract contained 30.0% oleuropein equivalent to 5.1% recovery (g/100 g of olive leaves). However, the process required high amounts of CO2 and co-solvent leading to a costly procedure.

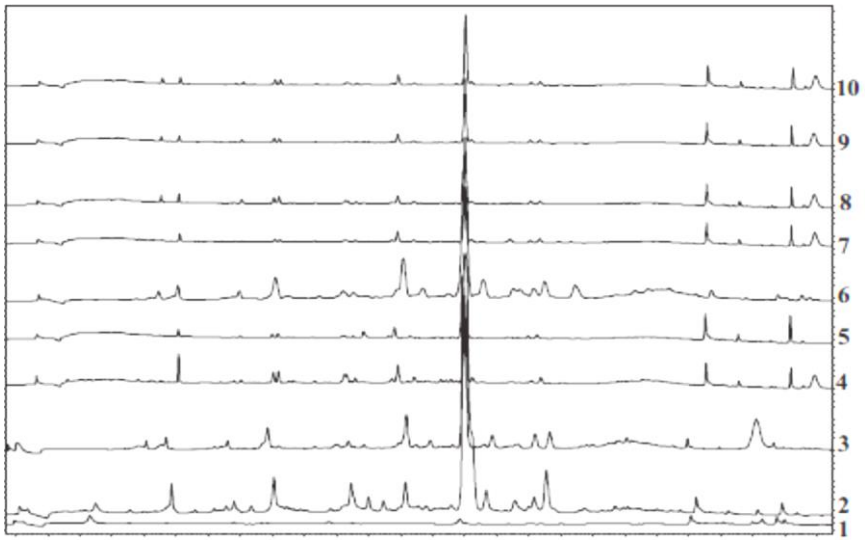


Figure 27. Overlay of HPLC chromatograms of extracts obtained from olive leaves: 1. SFE with 5% ethanol, 2. SFE with 20% ethanol, 3. PLE with ethanol 100% at 40 ◦C, 4. PLE with water 100% at 50 ◦C, 5. PLE with water/ethanol 40:60 at 50 ◦C, 6. PLE with subcritical ethanol 100% at 115 ◦C, 7. PLE with subcritical water 100% at 150 ◦C, 8. PLE (after SFE) with water 100% at 50 ◦C, 9. PLE (after SFE) with water/ethanol 40:60 at 50 ◦C and 10. PLE (after SFE) with subcritical water 100% at 150 ◦C. Major peak represents oleuropein.

Ratio[a]	Flow rate (kg/h)	P (MPa) in extractor	Co-solvent (wt%)	Dry extract (g)	Yield (w/w%)	Oleuropein (%)[b]	Recovery (%)[c]	DPPH (μg/mL)
120	9.6	30	5%	40.42	14.7	0.0	0.0	752.1
290	9.6	30	20%	46.75	17.0	30.0	5.1	113.9

[a] Solvent-to-feed ratio.
[b] (%) oleuropein in the dry extract.
[c] (%) oleuropein in olive leaves.

Table 6. Conditions of SFE for the extraction of oleuropein and obtained results

PLE was elaborated in order to comparatively study its effectiveness in the extraction process of the polar constituents from olive leaves. In order to maximize the recovery of oleuropein, water and ethanol were utilized. Ethanol (at 40 ◦C), water (at 50 ◦C), subcritical ethanol (at 115 ◦C), subcritical water (at 150 ◦C) and a water/ethanol 40:60 mixture (at 50 ◦C) were used. 6.0 g of sieved olive leaves were used in each case. The parameters of the extraction are depicted in Table 7. It was observed that the yields of the extracts were high and the parameters employed allowed the extraction of polar compounds. The highest yield (41.5%) was achieved with subcritical water. The application of high temperature during the extraction causes the decrease of the dielectric constant (polarity) of the water, resulting in parallel in a steady decrease in viscosity and surface tension, as well as in faster diffusivity characteristics. Qualitative characterization of the extracts by RP-HPLC showed that the (%) content and recovery of oleuropein were higher at the extract obtained with subcritical ethanol (16.4% and 4.3%, respectively). However, these percent-ages did not differ much from the ones obtained with subcritical water (8.3% and 3.4%, respectively), thus the use of water was also considered advantageous (Fig. 24). Although, ethanol proved again an effective extractant for oleuropein, a procedure performed with 100% ethanol is not easily applicable due to its high-cost.

T (◦C)	Heat (min)	Pre-heat (min)	Solvent	Static time (min)	Cycles	Dry extract (g)	Yield (w/w%)	Oleuropein (%)[a]	Recovery (%)[b]	DPPH (μg/mL)
40	5	1	EtOH	10	2	1.57	26.2	14.5	3.8	123.4
50	5	1	H_2O	10	2	0.93	15.5	10.7	1.7	128.0
50	5	1	H_2O/EtOH (40:60)	10	2	1.60	26.7	7.3	1.9	124.0
115	5	1	EtOH	10	2	1.58	26.4	16.4	4.3	134.7
150	5	1	H_2O	10	2	2.49	41.5	8.3	3.4	132.5

[a] (%) oleuropein in the dry extract.
[b] (%) oleuropein in olive leaves.

Table 7. Conditions of PLE for the extraction of oleuropein and obtained results.

After experimentation with both techniques separately, their combination was investigated based on the competence of each technique. The preference of SC-CO2 for the extraction of the undesirable apolar and lipophilic substances (fatty substances, waxes and chlorophylls) from the leaf matrix led to its elaboration for defatting of the initial material, while PLE with polar solvents was used for the isolation of the polar compounds of interest. The addition of co-solvent, specifically ethanol at percentage high enough to assist in the removal of lipophilic compounds but not permit-ting the extraction of oleuropein was considered essential. Initially, olive leaves were placed at the SFE apparatus for extraction with CO2. One of the most important advantages of SFE is the possibility of changing operational conditions to facilitate the extraction of specific compounds. As a consequence, in the present study, the procedure proceeded with gradual increase of the pressure in order to achieve the fractionation of the extract and the acquisition of a defatted residue. 412.4 g of tree olive leaves were placed inside the 1 L extractor of the apparatus. TLC of the extracts obtained and co-chromatography with standard compounds permitted a quick preview of their chemical content. The details and the parameters of the trial are depicted in Table 3. Initially, low pressures (12, 15 MPa), accounting to low CO2 density and solvating power, were selected in order to extract low molecular weight compounds. During this step of the extraction a pure white powder was deposited in the separators. Analysis with TLC revealed that the sample was dominated by waxes. As the extraction proceeded, the extract was enriched with a yellow, thick substance, which was soluble only in the supercritical state, and thus it was solidified on the walls of the separators. A subsequent rinse of the separators with ethanol was mandatory in order to remove them from the interior walls. Then, higher pressures were applied (20, 25 MPa), keeping the temperature constant aiming to maximize the density and thus the solvating power of CO2 in order to remove higher molecular weight compounds. The extract had an intense yellow color and analysis indicated the presence of fatty acids and a small amount of carotenoids. Under these conditions the extract was again not completely removed from the separators and a subsequent rinse with ethanol was necessary. Since the yield of the extraction was not significantly high, a further increase of pressure, also result-ing in polarity modification was applied in the system in order to improve the yield and the selectivity of the process. Pressure of 30 MPa was applied and the mobile phase was modified with ethanol (1–5%). The addition of the co-solvent was again gradual and cautious since the point that the

majority of the lipophilic compounds along with chlorophylls would be removed from the residue and oleuropein would be extracted, should be identified. The extract had a green color indicating the presence of chlorophylls. TLC analysis revealed that terpenoids were also obtained and more specifically oleanolic acid, maslinic acid and β-sitosterol. Sequentially the amount of co-solvent increased to 10%. Extract yield increased with co-solvent administration, compared to the previous samples. Of interest was the isolation of hydroxytyrosol and oleuropein only in traces. It was decided that the extraction should stop at this point and the residue was placed into the PLE apparatus in order to continue with the extraction of the polar compounds. Three different methodologies were applied, focused on maximizing the recovery of oleuropein:water (at 50 ◦C), sub-critical water (at 150 ◦C) and a water/ethanol 40:60 mixture (at 50 ◦C). 6.0 g of the SFE's residue were used in each case while the parameters are depicted in Table 8. It was observed that the residue of the SC-CO2, which was processed afterwards with PLE, gave really high yields (33.5–44.1%) of dry extracts. These results were due to the removal of the fatty components from the leaf matrix. Oleuropein was detected at t_R = 30.4 min as the major peak in all extracts analyzed. The SFE residue extracted with subcritical water gave the highest yield (44.1%) and recovery of oleuropein (4.6%) (Fig. 20). Apart from these, the removal of non polar compounds achieved with SFE and the decreased amount of sugars obtained with subcritical water gave to the final extracts additional desirable features. The comparison of these results with the ones obtained after the application of the two techniques separately showed that the serial combination of SC-CO2 modified by ethanol (up to 10%), with PLE especially subcritical water, can be advantageous since it offers high yield extracts with high recovery of oleuropein. SC-CO2 modified with ethanol (20%) and PLE with ethanol (100%) can also lead to the extraction of oleuropein but a high percentage of ethanol is necessary.

Extractor					
Ratio[a]	Flow rate (kg/h)	P (MPa)	Co-solvent (wt%)	Dry extract (g)	Yield (w/w%)
45	5.0	12	–	4.02	0.97
70	5.0	15	–	0.06	0.01
100	5.0	20	–	0.11	0.03
130	5.0	25	–	1.18	0.29
160	5.0	30	–	0.08	0.02
350	5.0	30	1	6.00	1.45
520	5.0	30	3	8.62	2.09
610	5.0	30	5	4.24	1.03
660	5.0	30	10	4.37	1.06

[a] Solvent-to-feed ratio.

Table 8. Conditions of SFE for the extraction of lipophilic compounds and obtained results.

T (◦C)	Heat (min)	Pre-heat (min)	Solvent	Static time (min)	Cycles	Dry extract (g)	Yield (w/w%)	Oleuropein (%)[a]	Recovery (%)[b]	DPPH (μg/mL)
50	5	1	H_2O	10	2	2.16	33.5	9.9	3.3	138.6
50	5	1	H_2O/EtOH (40:60)	10	2	2.30	35.7	9.5	3.4	145.3
150	5	1	H_2O	10	2	2.84	44.1	10.4	4.6	127.3

[a] (%) oleuropein in the dry extract.
[b] (%) oleuropein in olive leaves.

Table 9. Conditions of PLE for the extraction of oleuropein from the defatted residue (after SFE) and obtained results.

Lastly the extracts were evaluated for their radical scavenging activity with the DPPH assay. The application of the DPPH test demonstrated that the extracts exhibited radical-scavenging activity and the results appear in Tables 6, 7 and 9. As observed all the extracts showed good antioxidant activity without significant variations. The extracts obtained by SFE modified with 20% ethanol (Table 6) and PLE with 100% ethanol (40 ◦C) and water/ethanol 40:60 (50 ◦C) (Table 7) showed the best activity. It is hypothesized that the organic solvent causes the extraction of other categories of compounds like flavonoids which also contribute to the antioxidant properties. The extracts obtained from SFE (up to 10% ethanol) had no significant antioxidant activity, something expected since they contained no antioxidant substances. Previous studies refer that the solvent type can affect the antioxidant activity of olive leaf extracts and methanol is recommended as the solvent of choice. In the present study we suggest that an extraction performed under carefully investigated conditions produces extracts with high oleuropein content and thus good antioxidant activity even when it is performed with a non toxic solvent, like water.

1.2.1.4) Conclusions

In this first part of the PhD experimental part, an efficacious, simple and selective procedure for extracting olive leaves was introduced, using super/subcritical fluids. It is based on the elaboration of contemporary techniques using “green” approaches. Compared to previous studies, the present research proposes the combination of two “green” techniques that could provide the optimum extraction of the initial material by carefully selecting the conditions promoting the recovery of the desirable compounds. The removal of lipophilic compounds, which were of minor importance in the present study since they do not contribute to the antioxidant activity of the plant, succeeded with SC-CO2, led to a residue rich in phenolic com-pounds, which was extracted sequentially with subcritical water in PLE. The result was high yield extracts, rich in oleuropein that exhibited high radical scavenging activity. SFE and PLE individually provide high oleuropein content, however the need for high per-centages of co-solvent/solvent is not cost effective. Furthermore, PLE with water or water/ethanol provide high extraction yields but moderate recoveries of oleuropein. The present study proposes a “green” extraction procedure which affords extracts rich in oleuropein, while in parallel minimizes extraction time, uses no harsh organic solvents and eliminates problems in extraction caused by lipids and chlorophylls. Of major importance is that the extract finally obtained is characterized by desirable traits and could be used for human consumption. The information acquired from this study will be used to optimize the extraction process accordingly and make feasible the industrial implementation of the proposed method.

1.2.2) Design optimization study of the extraction of olive leaves performed with pressurized liquid extraction using response surface methodology.

1.2.2.1) Aim of the study

The main aim of the present study is to establish an optimized, fast, highly sensitive and "green" method for the acquisition of olive leaves extracts with specific desired properties: high oleuropein content and/or high antioxidant activity, combined with maximum extraction yield. For this purpose a two-stage optimization process was designed. Firstly, a Plackett-Burman design was applied in order to determine the statistically significant parameters that affect a PLE process. Subsequently, a Central Composite Design (CCD) was implemented in order to maximize the responses (extraction yield, oleuropein content and radical scavenging activity) through the construction of a response surface.

1.2.2.2) Materials and methods

1.2.2.2a) Solvents and Reagents

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) reagent was obtained from Sigma-Aldrich. Carbon dioxide was supplied by Air-Liquid Hellas in cylinders. Absolute ethanol was obtained by Carlo Erba Reagents. Methanol (MeOH) HPLC grade was obtained from Merck KGaA, Darmstadt and acetic acid 100% glacial from Riedel-de Haën. HPLC-grade water was obtained by double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, MSU).

1.2.2.2b) Plant material

Olea europea leaves var. Koroneiki were collected in 2009 at the region of Attica, dried in a well ventilated shady place and subsequently stored in a dark room. Before the extraction the leaves were grinded using an AllenWest type SCIS grinder with a sieve of 3mm.

1.2.2.2c) Pressurized liquid extraction (PLE)

A Dionex Accelerated solvent extraction (ASE) 300 System (Dionex, Sunnyvale, CA) with 100 mL stainless steel vessels was used for the pressurized liquid extraction. Specifically 7.0 g of grinded olive leaves powder were placed into the tubular extraction cell. The leaves were then placed into the carousel and the samples were extracted under the specified conditions. Constant pressure was applied at 1500 psi. The extraction procedure was performed in the static mode (Tables 1 and 2). Finally, the extracts were evaporated to remove ethanol using a rotary evaporator (Buchi Rotavapor R-200) at 45 °C and subsequently submitted to lyophilization.

1.2.2.2d) HPLC analysis

The quantitative determination of oleuropein was performed with a Thermo Finnigan HPLC system (Thermo Finnigan, San Jose, CA) coupled with a Spectra System UV6000LP PDA detector. A two-solvent gradient method was used with solvent A. H2O+1% acetic acid (v/v) and B. MeOH. The flow rate was set at 1 mL/min and the following elution program was applied: 0-2 min linear gradient to 5% B; 2-10 min linear gradient to 25% B; 10-20 min linear gradient to 40% B; 20-30 min linear gradient to 50% B; 30-34 min 50% B isocratic; 34-45 min linear gradient to 90% B; 45-50 90% B isocratic; 50-60 min linear gradient to 100% B; 60-65 min 100% B isocratic. Standard solution of oleuropein was prepared in H2O/MeOH 50:50. The separation was performed at 25 °C and the injection volume was 20 μL. The detection was performed at 248 nm and the column used was Supelco Analytical Discovery HS C18 (25cm x 4.6 mm, i.d., 5.0 μm).

1.2.2.2e) Antioxidant activity – DPPH method

The assay was based on the publication of Lee et al. (1998) [9] as follows: 12.4 mg of DPPH were dissolved in 100 mL of ethanol. 190 μL of this DPPH solution were mixed with 10 μL of the oleuropein-enriched extracts dissolved in DMSO. The mixture was left for incubation at 37 oC for 30 min in the dark and the absorbance at a wavelength of 517 nm was measured. Initially, the DPPH scavenging activity was estimated in four different concentrations (25, 50, 100 and 200 μg/mL) for each extract and afterwards the IC50 was calculated. All the experiments were performed in triplicate using 96 well plates employing an Infinite m200 pro TECAN reader (Tecan Group, Männedorf Switzerland)

1.2.2.2f) Experimental design

The application of the experimental design allows studying the influence of several process parameters on one or more responses of the design. Design of experiments (DoE) is able to determine interactions between parameters and predict extraction conditions using a minimum number of experiments [Silva et al., 2007]. More specifically, a central composite design (CCD) is an experimental design, useful in response surface methodology, for building a second order (quadratic) model for the response variable without needing to use a complete three-level factorial experiment. It consists of three distinct sets of experimental runs:

1. A factorial (perhaps fractional) design in the factors studied, usually performed at two stages.
2. A set of center points, experimental runs at the center values of all factor ranges. These points are often replicated in order to improve the precision of the experiment and predict curvature.
3. A set of axial points, experimental runs performed at the alpha values in order to maintain the rotatability of the design [Kassama et al., 2007].

The main aim of the present study is to establish an optimized, fast, highly sensitive and "green" method for the acquisition of olive leaves extracts with specific desired properties: high oleuropein content and/or high antioxidant activity, combined with maximum extraction yield. For this purpose a two-stage optimization process was designed. Firstly, a Plackett-Burman design was applied in order to determine the statistically significant parameters that affect a PLE process. Subsequently, a Central Composite Design (CCD) was implemented in order to maximize the responses (extraction yield, oleuropein content and radical scavenging activity) through the construction of a response surface.

All DoE procedures were performed with the use of the free trial version of Design Expert® - 8 (StatEase, Inc, Minneapolis, MN). A Plackett-Burman factorial design was applied using eleven parameters at two levels employing five center points. A central composite design (CCD) approach has been used for modeling the responses obtained by the Plackett-Burman design. Each parameter is estimated at five levels, namely ±1, ±α, and one center point replicated five times. Numerical optimization has been applied for determining the best solutions.

1.2.2.3) Results

1.2.2.3a) Screening design

In the present work, a full-set of optimization procedure has been employed. Due to the number of parameters involved in PLE extractions, each one of them reporting different behavior on the response of interest, a screening experimental design approach was initially performed in order to determine the most influential parameters, in terms of yield, oleuropein content and radical scavenging activity of the dry extract. The aim was to minimize the number of experiments needed to optimize the responses under evaluation. A Plackett-Burman factorial design was applied in order to explore a n-dimensional experimental space using n+1 experiments. Such experiments are regularly used when the parameters potentially contributing to the model are more than seven. The main characteristic of such designs is that the main effects are orthogonal between them and they are only partially aliased with higher order interactions, which differentiates them from the resolution three-fractional factorial designs (main effects are aliased with two-factor interactions). In the present case, 11 factors have been employed leading to a total of 12 experiments (n+1). Each factor's values (low and high) are equally distributed throughout all the experiments, namely in 6 (+1) and in 6 (-1) levels. Each main effect can be determined by the following equation:

$$\text{Effect} = 1/6\ [\Sigma\ (y{+}1\ \text{level}) - \Sigma\ (y{-}1\ \text{level})] \qquad (1)$$

The Plackett-Burman screening procedure indicates the main factors influencing the measured responses. In more details, eleven parameters have been considered in the screening design, eight of them being actual extraction settings: %EtOH content in the solvent mixture (EtOH), temperature of the extraction(Temp.), Static time, cycles of repeated extractions(Cycles), volume of solvent flush at the end of each extraction cycle(Flush), purge duration(Purge), pre-heat duration (Preheat) and cell volume (Cell vol.) and three of them being "dummy" variables. Low and high value were decided for each parameter, taking into account the apparatus limitations, as well as previous experience in PLE. The responses that were used in order to evaluate the influence of the extraction parameters were the extraction yield, the oleuropein content of the extract and the radical

scavenging activity. The recovery of oleuropein from the olive leaves is defined as the weight of oleuropein that was obtained from the extraction of 100 grams of olive leaves and is calculated as follows:

$$\text{Recovery} = \frac{[(\%\ \text{yield}) * (\%\ \text{oleuropein})]}{100} \quad (2)$$

where % yield is the OTL extraction yield in dry matter and % oleuropein is the content of the dry extract in pure substance. The designed series of experiments are summarized in Table 10, along with the results for the various responses.

EtOH	**Temp.**	**Static time**	**Cycles**	**Flush**	**Purge**	**Pre-heat**	**Cell Vol.**	**Dummy1**	**Dummy2**	**Dummy3**	**Yield**	**Oleuropein**	**Recovery**	**DPPH**
Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1	Response 2		Response 3
%	°C	Min	No	%	sec	sec	ml				%	%		IC_{50} (μg/ml)
100	190	25	1	40	60	180	33	1	1	-1	50,7	10,6	5,4	131,0
0	40	5	1	40	60	60	100	-1	-1	-1	26,6	11,2	3,0	157,5
100	40	5	1	100	60	180	100	-1	1	1	24,3	14,3	3,5	125,9
0	40	25	1	100	180	60	100	1	1	-1	29,9	10,7	3,2	126,0
100	40	25	3	40	180	180	100	-1	-1	-1	31,4	15,7	4,9	118,4
0	190	25	3	40	60	60	100	-1	1	1	53,0	11,4	6,0	159,1
100	40	25	3	100	60	60	33	1	-1	1	33,4	18,4	6,1	151,3
0	190	25	1	100	180	180	33	-1	-1	1	53,3	10,0	5,3	138,5
100	190	5	3	100	180	60	33	-1	1	-1	51,3	12,0	6,2	138,2

100	190	5	1	40	180	60	100	1	-1	1	46,0	9,3	4,3	122,5
0	40	5	3	40	180	180	33	1	1	1	30,3	11,7	3,5	146,9
0	190	5	3	100	60	180	100	1	-1	-1	49,9	10,7	5,3	144,7

Table 10. Experimental values of responses for screening design of experiments (Plackett-Burman factorial design).

Statistical analysis was performed by means of analysis of variance (ANOVA). After inspection of the normal probability plot, seven parameters have been rendered significant with p-values of the F-test lower than 0.05. The adequate precision value that has been calculated is 191, which is in large excess than the proposed value of 4. This shows that the signal to noise ratio is much smaller than the actual effect size.

The influence of the temperature, the static time and the cycles account for more than 87% of the total effect on the % yield response. In order to identify the main effects, the Pareto chart for the influence on the studied parameters has been used. As shown in Fig. 25, concerning the yield of the extraction, only 3 parameters are statistically significant at a level of p=0.05 and lie above the t-value limit: temperature, static time and extraction cycles. The results for the oleuropein content have been treated similarly. As it can be seen from the Pareto chart for oleuropein in Fig. 26, for the oleuropein content also 3 -but partially different- parameters were significant and lie above the t-value limit: temperature, EtOH (%), and extraction cycles.

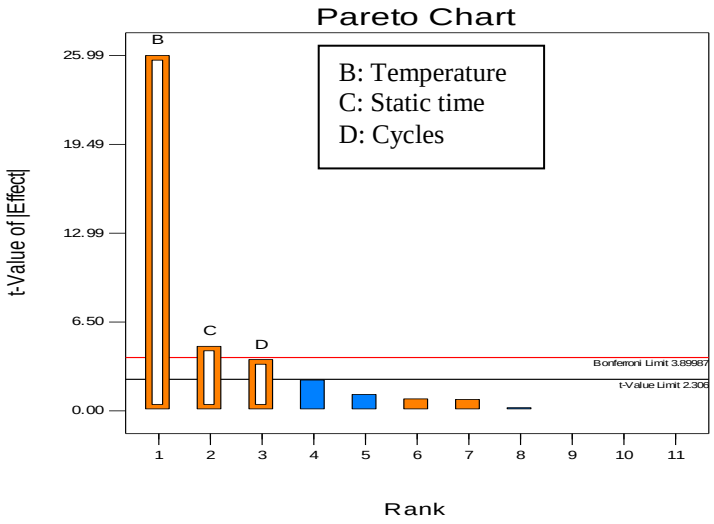


Figure 28. Pareto chart for the extraction yield

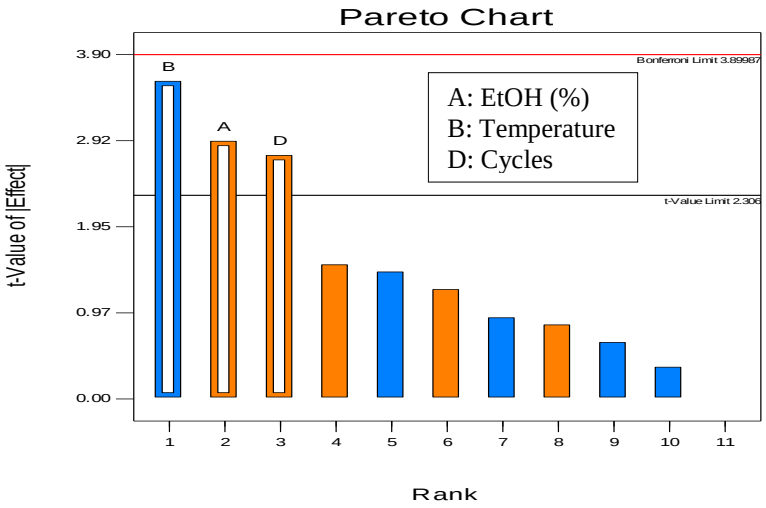


Figure 29. Pareto chart for the % oleuropein content

Regarding the radical scavenging activity, no statistically significant model could be fitted at a level of p=0.05. This fact is demonstrated in the Pareto chart for the radical scavenging activity (Fig. 27) where all studied parameters lie under the t-value limit. Thus, no parameters have been suggested to be included in the optimization procedure.

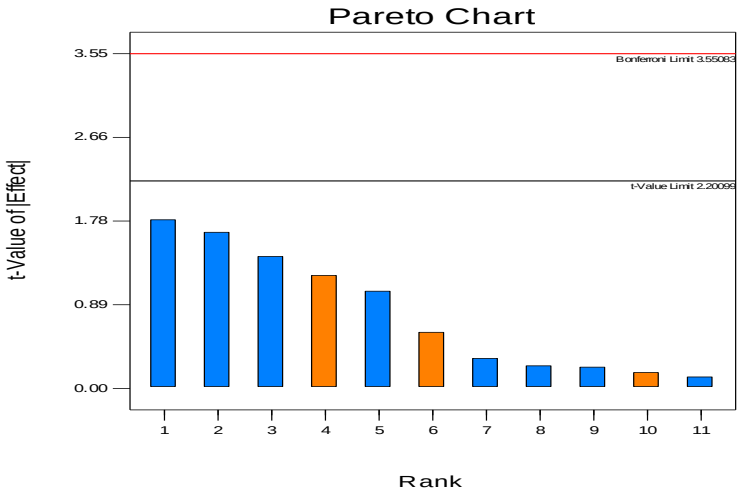


Figure 30. Pareto chart for the DPPH antioxidant activity

The ANOVA table confirms the significance of the model in each case as well as of its individual factor at the level of p=0.05. The normal probability test does not indicate the presence of any outliers.

1.2.2.3b) Optimization design

Response surface methodology has a dual aim: to find the optimum settings for the variables and to explore their performance over the whole experimental domain, including any interactions. The central composite design (CCD) approach was used for modeling the responses obtained by the Plackett-Burman design. Such an experiment employees the standard 2k factorial points originating from the apexes of a cube, along with 2k axially-spaced points. A variant of CCD is the circumscribed design in which the axial points are chosen so to allow rotatability, which ensures that the variance of the model prediction is constant at all points equidistant from the design center. The center points could be replicated allowing an estimation of the noise [Boutin et al., 2009, Brachet et al., 2000, Loukas et al., 2001 and Jemai et al., 2009].

Totally, four parameters were significant, namely temperature, static time, extraction cycles and EtOH (%), and should be optimized. A design of three parameters was desirable. Two out of the four parameters were common for extraction yield and oleuropein content. As the main objective of the project was to optimize the oleuropein recovery of the dry extract, the ethanolic composition of the solvents mixture (%EtOH) was considered as more important parameter than the static time, affecting more significantly the recovery of oleuropein. Thus, the three most important parameters were: EtOH (%), temperature and extraction cycles. A rotatable CCD design was employed for the optimization procedure. Two numerical [EtOH (%) and temperature both varied over 5 levels ±a, ±1 and 0] and one categorical (extraction cycles, varied over 2 levels) factor were considered, and thus the design comprised of 22 experiments. The experimental conditions along with the values for the responses are summarized in Table 11.

Run	A: Temperature	B: EtOH	C: Cycles	Yield	Oleuropein	Recovery	DPPH
	°C	%	No	%	%		IC_{50} (µg/ml)
1	115.0	0.0	3	32.90	16.00	5.26	141.6
2	115.0	50.0	3	36.70	21.00	7.71	127.6
3	115.0	50.0	3	27.90	21.30	5.94	124.7
4	115.0	50.0	3	30.00	18.10	5.43	124.9
5	40.0	50.0	1	37.10	15.60	5.79	138.0
6	168.0	85.4	1	42.90	20.70	8.88	133.4
7	62.0	85.4	1	16.70	20.80	3.47	105.6
8	115.0	50.0	1	23.80	22.90	5.45	131.3
9	115.0	50.0	1	23.90	22.80	5.45	127.7
10	62.0	14.6	1	14.70	18.40	2.70	167.6
11	168.0	14.6	3	28.30	13.50	3.82	132.9
12	168.0	14.6	1	37.30	20.60	7.68	150.8
13	62.0	85.4	3	19.70	19.70	3.88	133.5
14	190.0	50.0	1	21.60	31.80	6.87	128.7
15	168.0	85.4	3	48.30	19.00	9.18	127.4
16	115.0	50.0	1	23.85	26.55	6.33	141.2
17	115.0	100.0	1	25.40	15.50	3.94	144.7
18	62.0	14.6	3	18.40	17.50	3.22	157.7
19	190.0	50.0	3	53.90	12.70	6.85	138.2
20	115.0	0.0	1	21.60	11.30	2.44	157.7
21	40.0	50.0	3	14.60	16.80	2.45	140.1
22	115.0	100.0	3	27.40	17.30	4.74	135.2

Table 11. Values for the parameters and responses of the CCD design. Highlighted the trial presenting the optimal oleuropein recovery

The 5 variables (PLE extraction settings) that were not considered significant during the experimental design were set at their minimum level for the completion of these experiments. The measured responses were three: yield (%), oleuropein (%) and IC50 for DPPH. Four models were considered, namely linear, linear + two-factor interactions, linear + two-factor interactions + quadratic terms, linear + two-factor interactions + quadratic terms + cubic terms. The analysis of variance demonstrated that, concerning the extraction

yield, the model was linear (model p-value>0.0001) with temperature being the significant term (p-value>0.0001). The final equation in terms of actual factors for 3 cycles is:

% Yield =7.59278+ (0.18088***Temperature**) + (0.046838***EtOH**) (3)

In this case no outliers have been detected. For the oleuropein content the suggested model was linear + two-factor interactions + quadratic terms (model p-value=0.0483) with significant terms the cycles (p-value=0.0502), the interaction between temperature and cycles (p-value=0.0239) and quadratic term of EtOH (p-value=0.0177). The final equation in terms of actual factors for 1 cycle is:

Oleuropein%= 5.04014+(0.14259***Temperature**) + (0.25876***EtOH**- 2.66667E-005***Temperature****EtOH**)- (3.78148E-004 * **Temperature**^{2})-(2.42083E-003**EtOH**^{2}) (4)

In this case no outliers have been detected. Concerning the DPPH IC_{50} the suggested model was linear + two-factor interactions + quadratic terms (model p-value=0.0137) with significant terms the EtOH (p-value=0.0026), the interaction between the temperature and EtOH (%) (p-value =0.0302) as well as the quadratic term of EtOH (%) (p-value =0.0215). The final equation in terms of actual factors for 1 cycle is:

DPPH IC_{50}= 201.42512- (0.45028***Temperature**)- (1.40281***EtOH**)+ (4.21850E-003* **Temperature****EtOH**)+ (1.01731E-003***Temperature**)+(5.71896E-003***EtOH**^{2}) (5)

Similarly in this case no outliers have been detected. For all models the predicted studentized residual values did not exceed the value of 3, which signifies that the models are reliable.

As it can be seen in Table 2, among the trials that were implemented, the optimal oleuropein recovery (9.18%) was achieved in Run 15, which had a yield of 48.30% and an oleuropein content of 19.00%. This extract was further analyzed using HPLC-DAD and by performing co-chromatography of various standard compounds usually present in olive leaves extracts. The main peaks were identified, as can be seen in Figure 28. Apart from oleuropein, among the main compounds were identified hydroxytyrosol, caffeic acid, acteoside, rutin and luteolin.

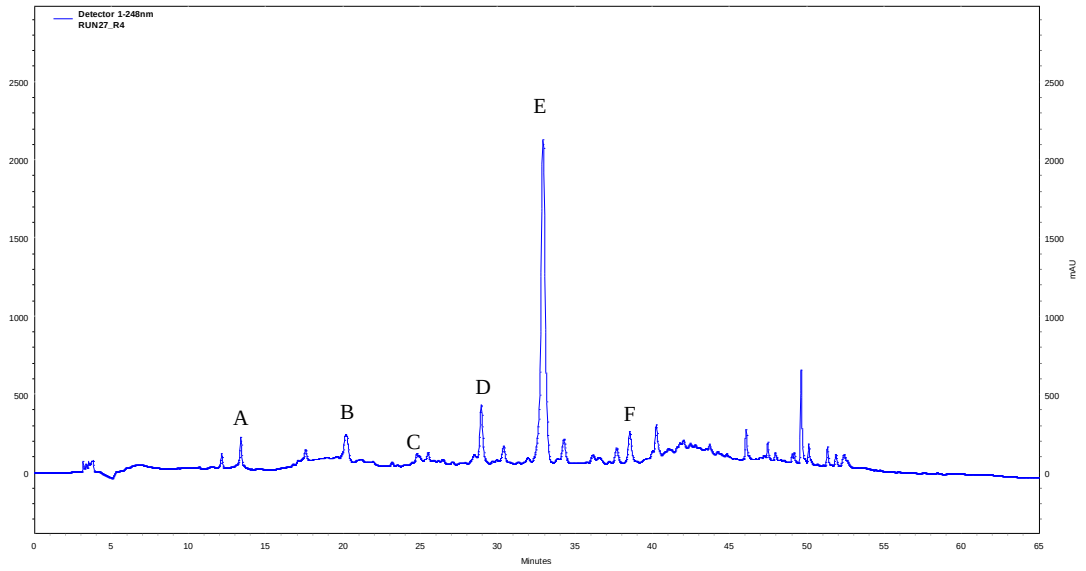


Figure 31. Phenolic compounds contained in olive leaves optimal extract. A: Hydroxytyrosol, B: caffeic acid, C: Acteoside, D: Rutin, E: Oleuropein, F: Luteolin

1.2.2.3c) Numerical optimization design

In order to find the optimal solutions of the aforementioned equations, numerical optimization was implemented for all responses. The limits used for the temperature variable were 40-190°C, for the EtOH content of the solvent mixture 0-100%, while the extraction cycles were considered as a categorical factor (1 and 3 extraction cycles). A simplex algorithm has been applied in order to obtain the maximum of each response of the 3-D space. Two solutions were found in each case with the desirability function for the yield being 0.16, for oleuropein content 0.22, for the DPPH 0.26 whereas the combined desirability for all three variables was 0.21.

The surface that exhibits how the response varies as a function of any two-factor interaction for each of the three measured responses lies in the respective 3D diagrams (Fig. 25-27). The obtained optimal solutions from the equations after the numerical optimization can be found in Table 12.

Experiment	Temperature °C	EtOH (%)	Cycles	Predicted Response
1	190.00	100.00	3	46.64[a]
2	190.00	89.35	3	46.15[a]
3	190.00	56.04	1	26.1[b]
4	82.82	56.54	3	21.0[b]
5	40.0	100.0	1	118.8[c]
6	175.28	39.68	3	125.9[c]

[a] Yield %
[b] Oleuropein content %
[c] DPPH (μg/mL)

Table 12. Optimal conditions for the various responses after simplex numerical optimization procedure

Furthermore, the model can provide possible solutions (i.e. experimental conditions) when targeting to specific response values. For instance, the possible solutions appointed by the model for an optimal yield with 25% oleuropein in the extract, are presented in Table 13.

Solution	Temperature °C	EtOH %	Cycles	Yield %	Oleuropein %	Desirability
1	190.00	77.27	1	39.01	25.0	0.737
2	190.00	34.85	1	37.02	25.0	0.697

Table 13. Optimal solutions for obtaining a maximum yield of an olive leaves extract containing 25% oleuropein

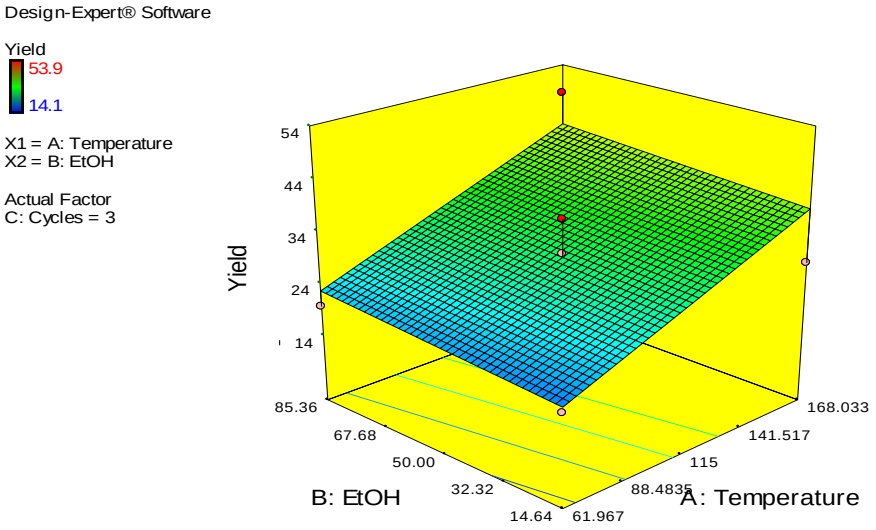


Figure 32. Response surface for extraction yield as a function of the EtOH (%) and Temperature

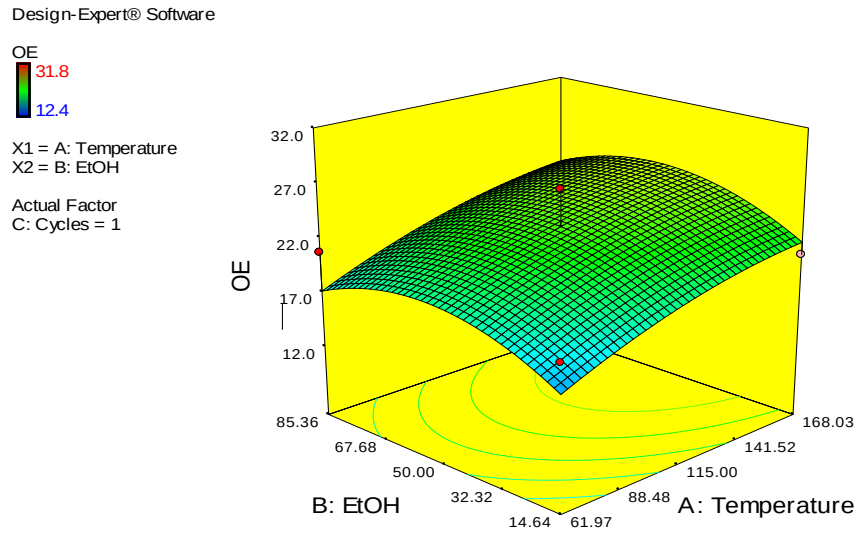


Figure 33. Response surface for oleuropein content as a function of the EtOH (%) and temperature

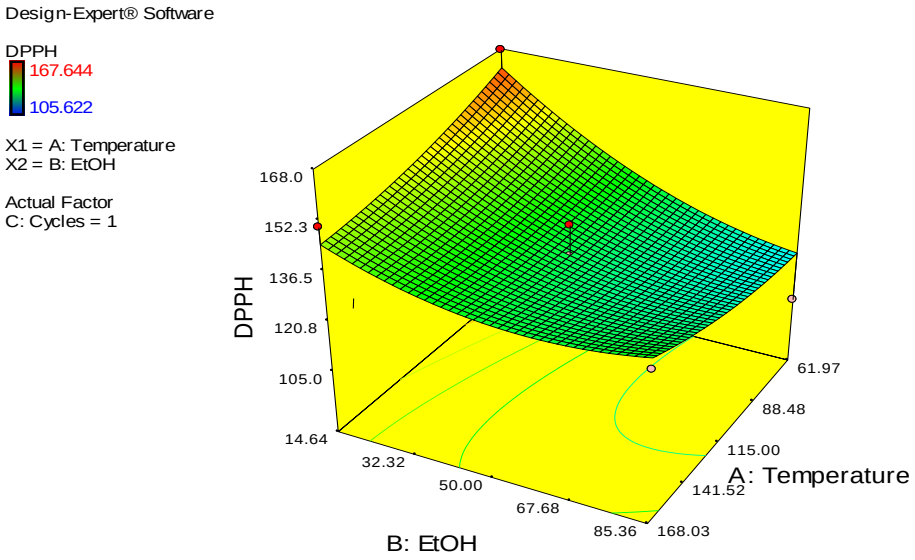


Figure 34. Response surface for DPPH IC50 as a function of the EtOH (%) and temperature

1.2.2.3d) Experimental validation of the model

In order to control the predictive capacity of the model, 2 different extraction procedures were set and each one was performed in triplicate. The experimental conditions were randomly selected, only under condition that they were not part of the screening or optimization design. One out of two extraction protocols lies in the darkest regions of the model regarding the experimental conditions, where predictability is supposed to be less important. Nevertheless, the measured responses in both experiments lay within a 95% confidence interval of the predicted values for % extraction yield and % oleuropein content of the extract. These results, presented in Table 14 below, confirm the predictability of the model constructed for the extraction of olive leaves.

Run	Significant condition values	Predicted % Yield (Mean ±CI 95%)	Observed % Yield (Mean ±SD)	Predicted % oleuropein (Mean ±CI 95%)	Observed % oleuropein (Mean ±SD)
1	85% EtOH, 120ºC, 1 cycle	26.71 ± 12.35	27.05 ± 0.92	21.90 ± 6.80	22.41 ± 2.35
2	100% H_2O, 40ºC, 3 cycles	17.17 ± 12.35	21.05 ± 2.43	20.20 ± 6.80	20.54 ± 2.71

Table 14. Comparison between the predicted and the observed values for the response variables, % yield and % oleuropein content

1.2.2.4) Discussion

In order to optimize the extraction procedure of olive leaves, a sequential optimization procedure has been followed: In the first step, the significant parameters affecting the desired measurable responses have been determined employing a Plackett-Burman design, whereas in the second step a numerical optimization procedure was elaborated in order to optimize them, using a CCD response surface methodology.

After analysis of the Plackett-Burman screening procedure it can be concluded that: The extraction yield is mainly influenced by 3 factors (in the order of statistical significance): temperature, static time and extraction cycles. The effect is positive in all three cases.

The oleuropein content of the extract is mainly influenced by 3 factors (in the order of statistical significance): EtOH (%)-positive effect, temperature -negative effect and extraction cycles-positive effect.

The DPPH scavenging activity is less influenced from the varied parameters than the other two responses. The parameter that mainly influences this response is the EtOH content of the solvent mixture, but it is not statistically significant. It has been observed that varying the extraction settings, diverse values for the IC_{50} were obtained, due to the presence of various polyphenols in the extract that contribute to the antioxidant activity. This should be explained by the fact that the vast majority of olive leaves' polyphenols, are being extracted in a wide range of experimental conditions. The fact that there is no significant variance in the IC_{50} against the DPPH free radical can be explained by the fact

that all the extracts contain an amount of oleuropein which is the main antioxidant but at the same time is not as extremely potent as for instance gallic acid, caffeic acid etc. Thus, variations in oleuropein content cannot significantly affect the antioxidant activity of the extract. Also, oleuropein's degradation product hydroxytyrosol which is very likely to occur in significant concentrations under certain conditions (prolonged heating and/or hydrolysis) demonstrates better antioxidant activity [Jemai et al., 2009]. The fact that the antiradical activity of the extracts was not correlated in a statistically significant manner with the total phenolic content of the leaves' extracts, oleuropein being the main phenolic in olive leaves, has also been observed in previous works. It is suggested that interactions among the various constituents are likely to define the antioxidant behavior of the extracts [Mylonaki et al., 2008].

Further on, the determination of the optimal conditions aiming to maximization of the responses by applying and analyzing a CCD led to the following conclusions: For higher extraction yield (calculated 46.64 ± 6.30%) the scheme proposed requires a temperature of 190 ºC, with 100% EtOH solvent for 3 extraction cycles. For higher oleuropein content (calculated 26.1±3.47%) the scheme proposed requires a temperature of 190ºC, H2O/EtOH/ 44:56 solvent mixture and 1 extraction cycle. It was observed that there is an interaction term between EtOH (%) and temperature which can be explained by the fact that hydroalcoholic mixtures will undergo phase transition at a certain temperature, becoming a subcritical fluid. This, as it is known, lowers the polarity significantly leading to higher oleuropein recovery. The fact that the oleuropein content is diminished when the extraction cycles are 3 instead of 1, can be explained by the fact that oleuropein is likely to be hydrolyzed to its metabolite hydroxytyrosol after intense and prolonged heating. For higher antioxidant activity (calculated DPPH IC_{50} 118.8±9.20 μg/ml) the proposed scheme requires a temperature of 40 ºC, with 100% EtOH and for 1 extraction cycle.

1.2.2.5) Conclusions

Pressurized liquid extraction has been applied in order to increase the extraction yield of Olea europea leaves, the oleuropein content of the extracts as well as their antioxidant activity. Response surface methodology was used to decide on the significant terms as well as to estimate and optimize the experimental variables. The maximum yield obtained was 46.64%, the maximum oleuropein content was 26.1% in the leaves' extract and the best IC_{50}

was 118.8 μg/ml. The quality control of the model concerning its predictability was very satisfactory. Such studies could help towards the design of higher efficiency protocols for the isolation of bioactive substances from olive leaves. These extraction protocols can also be very useful in combinatorial green processes which could be implemented in large-scale separations.

1.2.3) Investigation for the richest source of hydroxytyrosol (HT) among various olive processing by-products. Development of a sustainable procedure for the recovery of hydroxytyrosol from table olive processing wastewater using absorption resin technology and hydrostatic countercurrent chromatography

1.2.3.1) Aim of the study

Valorization of by-products of industrial olive processing through the recovery of high added value compounds, such as polyphenols, represents a promising approach for diminishing the environmental impact of wastes and repositioning olive industry in highly competitive levels. This work had two main purposes:

1. Sourcing: collection of various by-products of olive processing and assessment of their content in HT, as well as its recovery through conventional and "green" extraction methodologies.
2. Research and development: Establishment of a viable and industrially applicable procedure for the recovery of HT from the most abundant natural source, the table olive processing wastewater (TOPW). Comparison and evaluation of two different extraction/enrichment methodologies. The optimal process was developed from lab to pilot scale. The overall experimental strategy is summarized in figure 32.

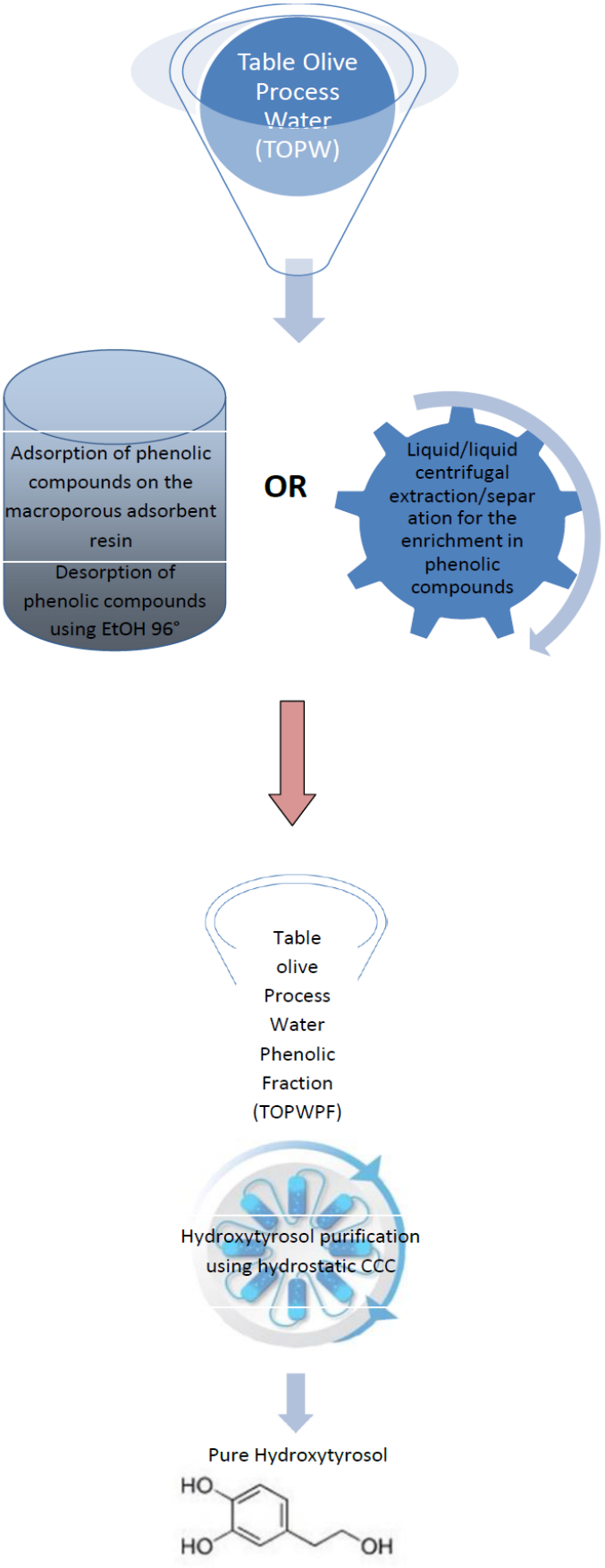


Figure 35. Overall experimental strategy for the extraction, enrichment and purification of hydroxytyrosol from table olive process water (TOPW)

1.2.3.2) Materials and methods

1.2.3.2a) Chemical reagents, standards and solvents

Methanol (MeOH), acetone (ACE), cyclohexane (c-Hex), ethyl acetate (EtOAc) and ethanol (EtOH) were of analytical grade (Merk, Darmstadt, Germany), and water (H2O) was used after distillation. Gradient HPLC-grade acetonitrile (ACN) was used for the HPLC-DAD analysis (Merk, Darmstadt, Germany). HPLC-grade water was obtained by double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, MSU). Amberlite XAD-4 resin (Rhom and Hass, France) was employed. Analytical thin layer chromatography (TLC) was performed on Merck pre-coated silica gel 60 F254 plates, and spots were visualized using UV light and vanillin-H2SO4 reagent. Hydroxytyrosol and p-coumaric acid standards (>98% pure) were purchased from Sigma-Aldrich.

1.2.3.2b) Olive processing by-products

The by-products obtained originate from three different sources located in the southern, central and northern Greece: Olive oil factory Renieris in Lakonia (http://www.renieris.eu/company_el.htm), as well as table olives and olive oil from Rovies Agricultural Cooperative in Evia(http://www.roviesolives.gr/Rovies/eisagogi.html). These by-products have been codified and are the following:

i) OLIVE PASTE NXHT2: (without olive wood). From Lakonia. It consists of the olive fruit mass after cold malaxation and centrifugal separation of the oil in a first step and of the wood in a second step. It contains around 3% of oil and 58.60% humidity, thus it must be treated relatively fast or frozen, or lyophilized (19.4% humidity). The paste is purchased by olive husk factories that produce the olive husk oil.

ii) RINSE WATER (after 2nd centrifuge) NXHT4: From Lakonia. It is the water that is used to refine olive oil (0.25 m3/ tn of oil). Its color is dark brown and probably needs to be treated relatively fast, otherwise to be refrigerated or frozen. This water contains no solids (no filtering needed) and it is disposed at the end of the process.

iii) OLIVE OIL MILL WASTEWATER (OOMW) NXHT15: From Evia. The classical three-phase centrifugal olive mill by-product.

iv) TABLE OLIVE PROCESSING WASTERWATER (TOPW) NXHT12: From Evia. For the sourcing step (1st step), one TOPW has been assessed, the Amfissis TOPW. Later on, for the extract

and pure HT process development, two different TOPWs, coming from two different olive varieties, were processed. Olive fruits of Kalamon variety (black olives) were debittered for a period of 4 months, using an aq. solution of 5% NaCl (w/v). This procedure yielded the Kalamon-TOPW. Olive fruits of Amfissis variety (brown olives) were debittered for a period of 4 months, using an aq. solution of 8% NaCl (w/v), yielding the Amfissis-TOPW. The TOPWs were provided by Rovies Agricultural Cooperative (Evia, Greece).

Figure 36. Table olive processing line in Rovies Agricultural Cooperative

Figure 37. Table olives from Rovies Agricultural Cooperative

1.2.3.2c) Resins

A 40 cm x 3 cm glass column packed with 50 mL of XAD-4 was used. The material passed through the column at a flow rate of 2 mL/min. The temperature remained at 20 °C throughout all of the experimental procedures. Adsorption capacities of resins at lab scale

were monitored in the elution solutions at 10 mL intervals by TLC. After reaching adsorptive equilibration, the resin column was first washed with H2O and then desorbed with EtOH. The contents of HT in desorption solutions were monitored by TLC every 10 mL. After desorption process, the desorbed solutions with high content of target compounds were collected, combined and then condensed to remove EtOH. Finally, the crude extract was obtained by drying at 40 °C to achieve constant weight. For pilot scale processes a column of 150 cm x 38 cm filled with 125 L of XAD-4 resin was used. Wastewater was fed into the resin using a 0.5 in. diameter PVC pipe using a Wilden diaphragmatic pump. The flow rate was 150 L/hr.

Figure 38. Pilot-scale macroporous resin columns (125 and 250 lt) in University of Athens, laboratory of Pharmacognosy

Figure 39. Pilot-scale solvent evaporation unit under vacuum (200 lt, QVF), University of Athens, laboratory of Pharmacognosy

1.2.3.2d) Liquid/liquid centrifugal extraction/separation

Rousselet-Robatel's BXP012 liquid/liquid centrifugal extractor/separator has been utilized for the development of an alternative to the resins method of enrichment in hydroxytyrosol, tyrosol and other TOPW phenolics. This apparatus has an internal diameter of 12mm, a useful capacity of 2.2 ml, a maximum rotor speed of 10350rpm, and a flow rate (for both phases) of 2 L/hr.

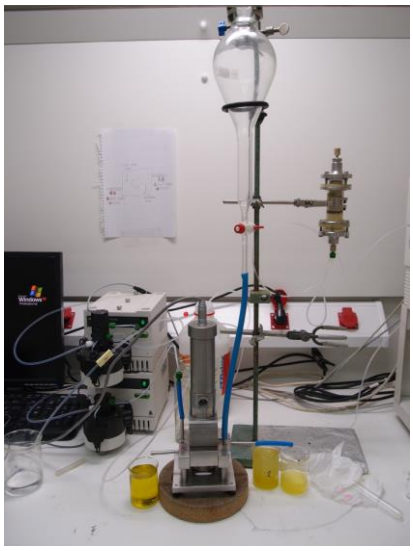


Figure 40. Rousselet-Robatel BXP012 liquid/liquid extractor

1.2.3.2e) CCC

Preparative separations were carried out on a Kromaton (Angers, France) FCPC® instrument equipped with a rotor of 1000 mL. The solvent was pumped through the system with a preparative pump LabAlliance, and the sample was injected via a 30 mL sample loop. Pilot separations were carried out on an SCCC 12.5 L ARMEN instrument (Vannes, France). The solvent was pumped through the system via AP1500 binary gradient pump and the sample was injected continuously. Chromatograms were recorded at 254 and 280 nm using a split towards UV/Vis DAD600 (200-600 nm) detector equipped with a prep flow cell and full automation was provided with ARMEN Glider software.

The suitability of the biphasic solvent systems was firstly evaluated by TLC, and then the two solvent systems giving the best apparent partition of HT were further analyzed by HPLC for the determination of partition coefficient values (Kd). This was performed as follows: an aliquot of TOPW extract (30 mg) was weighed into a 20 mL glass tube, 10 mL of the pre-equilibrated biphasic solvent system was added to the sample and shaken vigorously. The mixture was centrifuged at 2000 rpm for 1 min, and then 1 mL of each layer was taken out and evaporated to dryness. The residues were diluted in 1 mL of methanol, filtered on Nylon 0.45 mm and analyzed by HPLC-DAD. The Kd value was expressed as the peak area of the target compound in the stationary phase divided by the one in the mobile phase.

The biphasic solvent system consisting of c-Hex/EtOAc/EtOH/H2O (1:9:2:8, v/v/v/v) was selected. The solvents were thoroughly mixed in a separating funnel at room temperature prior to use, and the two phases of each system were separated after equilibration of the mixture. The TOPW extract was diluted in the lower phase of the solvent system prior to injection.

The hydrostatic CCC separation procedure developed as follows:

i) Preparative CCC: After filling with the stationary phase (upper phase), the rotation was set at 850 rpm and the mobile phase pumped through the system at a flow rate of 10 mL/min. 6 g of TOPWPPF were diluted in 30 mL of stationary phase and injected into the column before the elution step. Fractions (30 mL) were collected immediately after the injection of the TOPWPF extract, every 3 min. The CCC separation was monitored by TLC analysis of the fractions.

ii) Pilot-scale CCC: The preparative method was transferred to the SCPC 12.5 L instrument. After filling with the stationary phase (upper phase), the rotation was set at 850 rpm and the mobile phase pumped through the system at a flow rate of 50 mL/min with the use of an AP1500 binary gradient pump. 200-400 g of the TOPWPF were diluted in 225-450 mL of stationary phase and injected into the column before the elution step. Fractions (500 mL) were collected immediately after the injection of the TOPWPPF extract.

Figure 41. CCC with preparative column (Kromaton FCPC®, 1 lt) in University of Athens, laboratory of Pharmacognosy

1.2.3.2f) HPLC analysis

The quantitative determination of HT in the extracts, as well as the Kd of HT and tyrosol (TYR) in the CCC biphasic systems, were performed with a Thermo Finnigan HPLC system (Thermo Finnigan, San Jose, CA) coupled with a Spectra System UV6000LP PDA detector. A two-solvent gradient method was used with H2O (A) and ACN (B). The flow rate was set at 1 mL/min and the following elution program was applied: 0 min. 5% B, 0-10 linear gradient to 15% B; 10-15 isocratic 15% B; 15-35 min linear gradient to 25% B; 35-40 min isocratic 25% B; 40-70 min linear gradient to 90% B; 70-71 min linear gradient to 5% B; 71-90 min. isocratic 5% B. Standard solution of HT was prepared in aq. ACN 50% (v/v). The separation was performed at 25 °C and the injection volume was 10 μL. The detection was performed

at 280 nm and the column used was Supelco Analytical Discovery HS C18 (25 cm x 4.6 mm, i.d., 5.0 μm).

1.2.3.2g) NMR analysis

i) NMR parameters

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance III spectrometer (Bruker Biospin GmbH, Reinsteten, Germany) operating at 600.11 MHz for 1H and at 150.11 MHz for 13C, with a 5 mm inverse detection probe. The spectra were recorded in deuteromethanol (CD3OD) with all shift values (δ) referred to its residual 1H (3.33 ppm) signal and expressed in ppm. For NMR quantification, typically, 32 scans were collected into 32K data points over a spectral width of 9014.423Hz with a relaxation delay of 10 s, an acquisition time of 2.56 s and a flip angle of 90°. FID's were Fourier transformed with LB of 0.3 Hz. NMR processing for all samples included phase correction (performed manually for each replicate) and manual baseline correction over the entire spectral range was done with Bruker Topspin 3.2 prior to integration.

ii) Calibration solutions

Stock solutions were prepared containing 10 mg/mL of standard HT and 10 mg/mL of p-coumaric acid, as internal standard in CD3OD. From these stock solutions, six diluted solutions were prepared in order to obtain a calibration curve for HT. The spectra of HT as isolated from CCC were recorded after diluting 4 mg in 750 μL of CD3OD, 3.0 mg of p-coumaric acid, as internal standard. For each case (preparative and pilot), three replicate samples were analyzed.

1.2.3.3) Results and discussion

1.2.3.3a) Evaluation of the various raw materials and extraction/fractionnation techniques

The raw materials (olive processing by-products) priory described can be divided to solid RMs and liquid RMs.

Regarding the liquid RMs, both of them have been tested: NXHT4, NXHT15 and NXHT12. Quantification was first directly performed to the raw material. As it can be seen, NXHT12 (turquoise) contains significant amounts of Hydroxytyrosol in contrast to NXHT4 (black).

After quantification it was found that NXHT12 contains 0.1% HT(1 lt. contains 1000mg) while NXHT4 contains 0.002% (1lt contains 20mg). NXHT12 thus, is 50 times richer in the desired compound. NXHT4 on the other hand contains in excess another compound which, after LC-MS and NMR analysis, is likely to be also a phenolic compound. This compound though is likely to degrade during the adsorption/desorption process as it could not be found in the residue or in the final extract.

Thus, regarding the liquid RMs the richest in OH-Ty was found to be NXHT12 (50 times richer than NXHT4). Both NXHT12 and NXHT4 were submitted to adsorptive resin treatment for the concentration of the small phenolics molecules. The results are presented in the Table 15. The NXHT4 extract contained 1.97% HT and the NXHT12 extract contained 11.41% HT. The HPLC chromatograms of NXHT4 extract (black) and NXHT12 extract (turquoise) lie in Fig. 39:

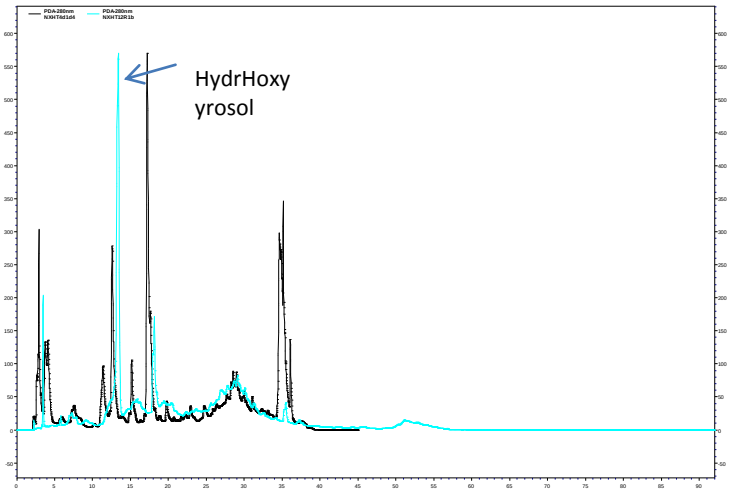


Figure 42 . NXHT4 (black) vs. NXHT12 (blue) chromatograms at 280nm (HPLC-DAD)

The adsorption/desorption process presents certain characteristics. The resin has a certain capacity to adsorb molecules due to their small size and their ability to form specific bonds with the matrix inside the pores. For each raw material, a study needs to be conducted in lab-scale simulation (height/bed volume ratio, flow rate etc.) for the process (adsorption AND desorption step) to be optimized, that is maximum volumes of raw material to be fed to the column without saturating it and minimum volumes of EtOH to be used in order to recover the totality of the adsorbed molecules. These studies have been conducted for both NXHT4 and NXHT12. In the first case the simulation did not work very well but in the second, and most important case, the volumes of raw material and EtOH as well as the appropriate flow rate were determined. It was observed that the significant quantities of

sodium chloride present in NXHT12 would not be retained from the resin and thus they were not found in the final extract.

Solid raw material is NXHT2 (olive paste without the oil and the wood) and was submitted to several extraction processes in order to asses, on the one hand its content in hydroxytyrosol, on the other hand the efficacy of the methods/processes tested to extract hydroxytyrosol from the raw material (recovery). In a first step an exhaustive extraction scheme was implemented, without considering important the industrial-scale feasibility or the "green" of the process, in order to calculate the content of the raw material in hydroxytyrosol and to have a method of reference for comparison of the various methods. Thus, a scheme of 4 consequent extractions of the same matter with MeOH and under sonication, followed by a microwave extraction to exhaust the residue, was applied. According to this, the content of NXHT2 in HT is 0.08% (1 kg contains 800mg). Subsequently, various extractions and/or fractionations were applied (presented in the same range as in Table 15) among which the most important were the following (along with the respective HPLC-UV chromatograms):

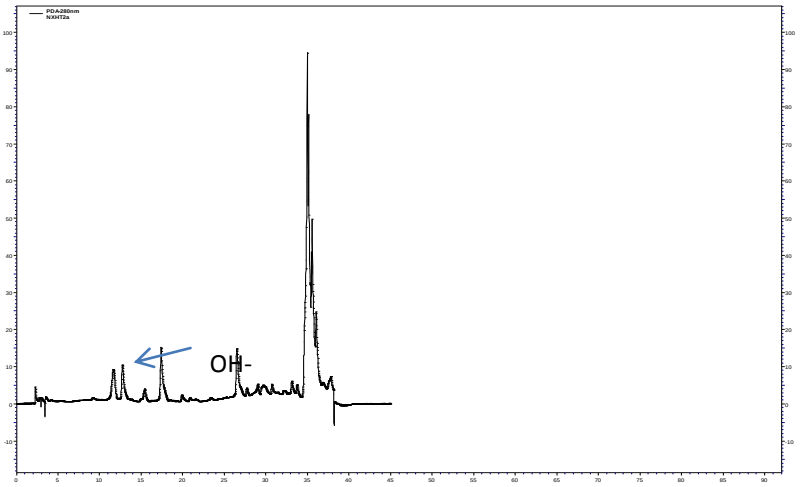


Fig. 43. NXHT2 SFE at 290 bar

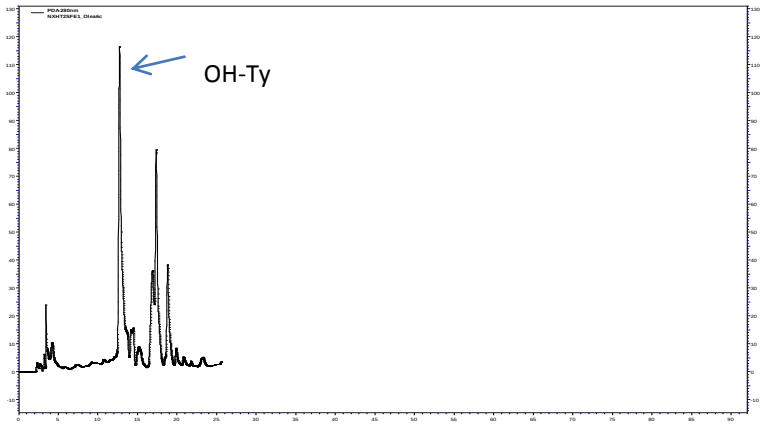


Figure 44. NXHT2 SFE at 290 bar with 3% of EtOH as a co-solvent

Figure 45. NXHT2 Lyophilization and SFE at 290 bar with 3% of EtOH as a co-solvent

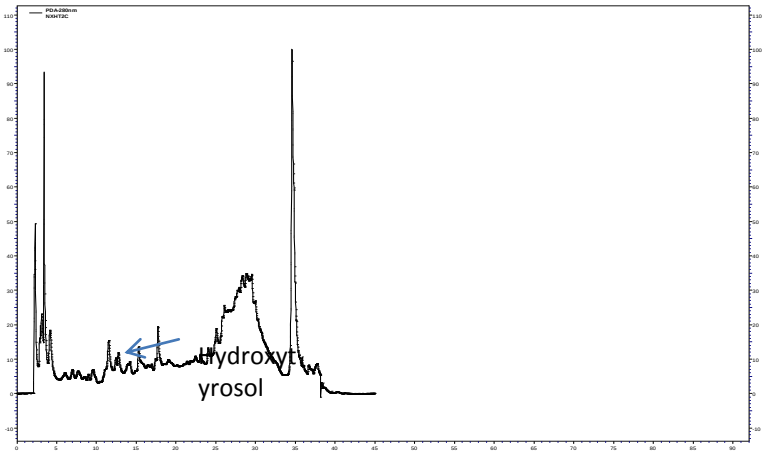


Figure 46. NXHT2 Maceration with water/boil in 100°C

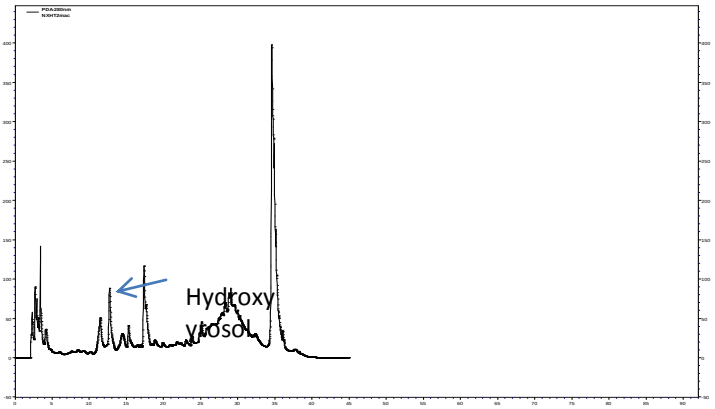


Figure 47. NXHT2 Maceration EtOH 96°, ambient temperature

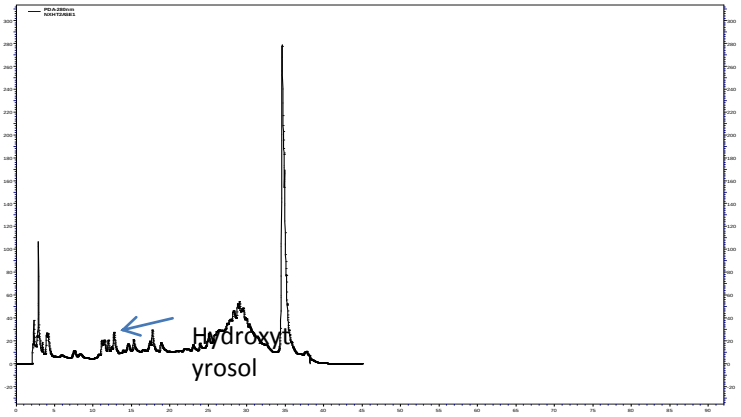


Figure 48. NXHT2 Pressurized liquid extraction (PLE) EtOH 96°, 70°C

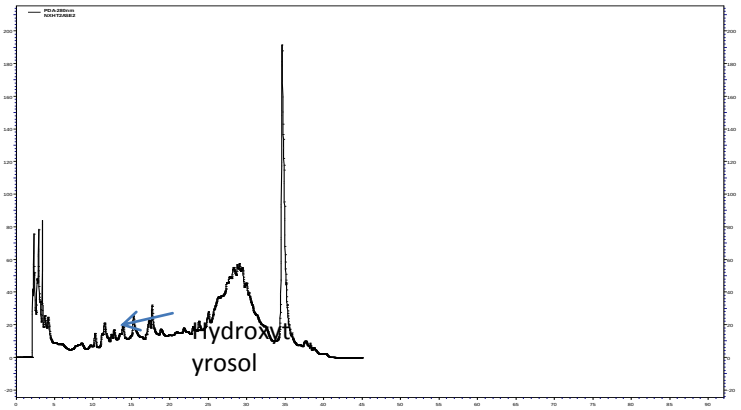


Figure 49. NXHT2 Pressurized liquid extraction(PLE) EtOH/H2O 50/50, 70°C

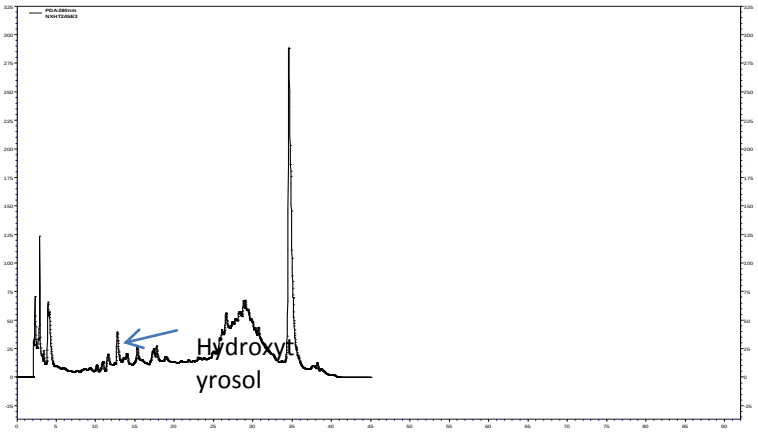


Figure 50. NXHT2 Subcritical water extraction 190 °C

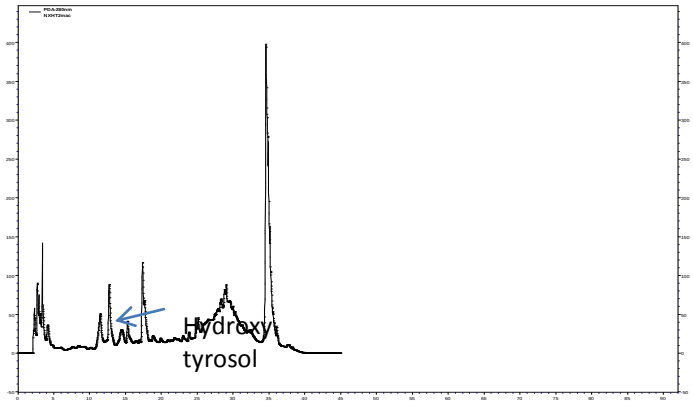


Figure 51. NXHT2 Maceration with EtOH/H2O and acidic environment (3 runs)

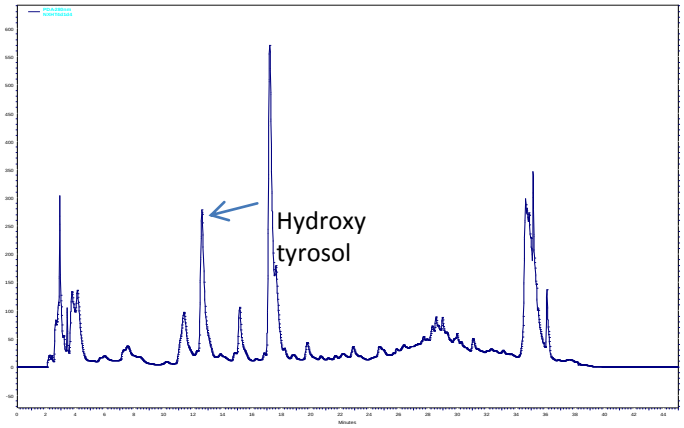


Figure 52. Adsorption Resin Technology (ART) with XAD-4 and EtOH for NXHT4

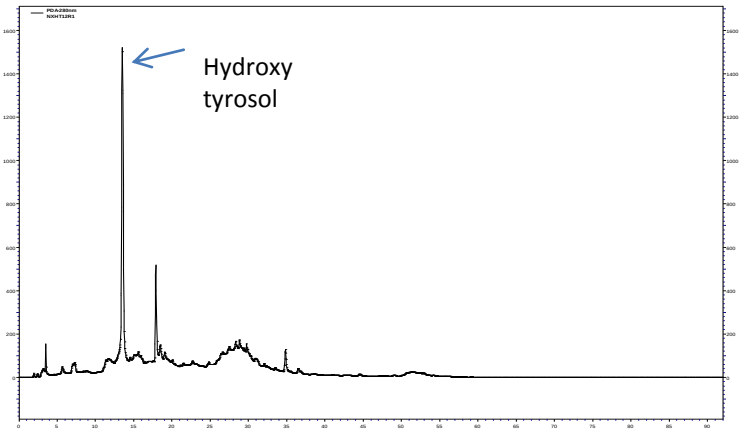


Figure 53. Adsorption Resin Technology (ART) with XAD-4 and EtOH for NXHT12

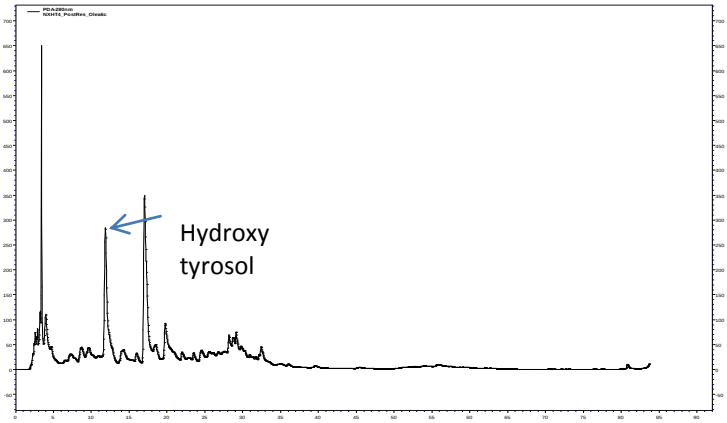


Figure 54. Adsorption Resin Technology (ART) with XAD-4 and EtOH for NXHT15

The extraction yields and hydroxytyrosol content in each case are presented in Table 15. Whenever there has been a fractionation, the total yield and active content for all fractions is indicated. The recovery is calculated as a ratio of HT obtained, in the respective extraction comparing to the exhaustive extraction scheme, the latter being considered to achieve absolute recovery.

RAW MATERIAL	SAMPLE CODE	EXTRACTION METHOD	SOLVENT	COMMENTARIES	DRY YIELD %	Σ(YIELD)	%OH-Ty in extract	% OH-Ty in RM	RECOVERY%	EXTRACT ASPECT
EXHAUSTIVE EXTRACTION SCHEME										
NXHT2 : OLIVE PASTE	NXHT2SON1	1st SONICATION	MeOH	CENTRIFUGAL SEPARATION	6.57		0.74			STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2SON2	2nd SONICATION	MeOH	CENTRIFUGAL SEPARATION	2.11		0.68	0.08 (1Kg contains 800 mg)		STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2SON3	3rd SONICATION	MeOH	CENTRIFUGAL SEPARATION	1.11	10.83	0.50		100	STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2SON4	4th SONICATION	MeOH	CENTRIFUGAL SEPARATION	0.60		0.87			STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2MW	MICROWAVE	MeOH	CENTRIFUGAL SEPARATION	0.44		0.71			STICKY PASTE
SFE										
NXHT2 : OLIVE PASTE	NXHT2a	SFE-RATIO 0-200	CO2	58.60% HUMIDITY/10% CELLULOSE/PACKING	0.92		0.1		1.15	OILY SEMISOLID
SFE/EtOH-FRACTIONNATION										
NXHT2 : OLIVE PASTE	NXHT2F1	SFE-RATIO 0-50	CO2+3% EtOH	58.60% HUMIDITY/10% CELLULOSE/PACKING	0.35		0.13			OILY SEMISOLID
NXHT2 : OLIVE PASTE	NXHT2F2	SFE-RATIO 50-100	CO2+3% EtOH		0.31	0.85	0.19		1.53	OILY SEMISOLID
NXHT2 : OLIVE PASTE	NXHT2F3	SFE-RATIO 100-150	CO2+3% EtOH		0.19		0.09			OILY SEMISOLID
SFE/EtOH										
NXHT2 : OLIVE PASTE	NXHT2SFE1	SFE RATIO 0-150	CO2+3% EtOH	58.60% HUMIDITY/10% CELLULOSE/PACKING	1.23		0.32		4.92	OILY SEMISOLID
LYOPHILIZATION/SFE/EtOH										
NXHT2 : OLIVE PASTE	NXHT2SFE2	LYOPHILIZATION/ SFE RATIO 0-150	CO2+3% EtOH	19.39% HUMIDITY/NO EXCIPIENT/NO PACKING	12.19		0.05		7.62	OILY LIQUID
MACERATION										
NXHT2 : OLIVE PASTE	NXHT2c	BOIL/MACERATION	H2O	FILTRATION PROBLEMS!	4.00		0.06		3.0	STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2MAC	MACERATION	EtOH 96°	CENTRIFUGAL SEPARATION	7.06		0.68		60.0	STICKY PASTE
PRESSURIZED LIQUID EXTRACTION										
NXHT2 : OLIVE PASTE	NXHT2ASE1	ASE	EtOH 96°,70°C		3.20		0.21		8.4	STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2ASE2	ASE	EtOH 96°/H20 50/50,70°C	LOW YIELD: PROBLEM	0.60		0.16		1.2	STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2ASE3	ASE	SUBCRITICAL H20 190°C		8.65		0.30		32.4	STICKY PASTE
CENTRIFUGAL EXTRACTION										
NXHT2 : OLIVE PASTE	NXHT2CF1	CENTRIFUGE	EtOH 96°		0.004					STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2CF2a	CENTRIFUGE	EtOH 96°		0.001	0.004				STICKY PASTE
NXHT2 : OLIVE PASTE	NXHT2CF2b	CENTRIFUGE	EtOH 96°		0.003					STICKY PASTE
MACERATION WITH ACID HYDROLYSIS										
NXHT4:RINSE WATER	NXHT4LH3	MACERATION IN pH=1	H2O/EtOH 50:50	CENTRIFUGAL SEPARATION	6.99		0.69			SEMISOLID PASTE
NXHT4:RINSE WATER	NXHT4LH1	MACERATION IN pH=1	H2O/EtOH 50:50	CENTRIFUGAL SEPARATION	6.84		0.50			SEMISOLID PASTE
NXHT4:RINSE WATER	NXHT4LH4	MACERATION IN pH=1	H2O/EtOH 50:50	CENTRIFUGAL SEPARATION	7.07		0.43			SEMISOLID PASTE
RESIN TECHNOLOGY-RINSE WATER										
NXHT4 : RINSE WATER	NXHT4							0.002 (1Lt contains 20 mg)		
NXHT4 : RINSE WATER	NXHT4a	XAD-4 RESIN	EtOH 96°	MIXING/STIRRING/FILTERING	0.60		0.17			STICKY PASTE
NXHT4 : RINSE WATER	NXHT4 (D1-D4)	XAD-4 RESIN	EtOH 96°	COLUMN/SATURATION STUDY : 11BV+2	0.03		1.97			STICKY PASTE
NXHT4 : RINSE WATER	NXHT4 XAD-4 RESIDUE	XAD-4 RESIN		BV	0.4		0.33			POWDER
RESIN TECHNOLOGY-RINSE WATER/INDUSTRIAL SCALE										
NXHT4:RINSE WATER	NXHT4INDini(DRIED)	XAD-4 RESIN	EtOH 96°	RAW MATERIAL			0.11			STICKY PASTE
NXHT4:RINSE WATER	NXHT4INDres(DRIED)	XAD-4 RESIN	EtOH 96°	RESIDUE/INDUSTRIAL COLUMN			0.04			POWDER
NXHT4:RINSE WATER	NXHT4INDext(DRIED)	XAD-4 RESIN	EtOH 96°	EXTRACT/INDUSTRIAL COLUMN			1.23			STICKY PASTE
RESIN TECHNOLOGY-DEBITTERING WATER										
NXHT12:DEBITTERING WATER	NXHT12						3.22	0.1 (1 Lt contains 1000 mg)		
NXHT12:DEBITTERING WATER	NXHT12R1	XAD-4 RESIN	EtOH 96°	COLUMN/SATURATION STUDY : 5BV+2 BV			11.41			HONEY-LIKE, LESS STICKY
NXHT12:DEBITTERING WATER	NXHT12R2	XAD-4 RESIN	EtOH 96°	COLUMN/SATURATION STUDY : 5BV+2 BV		1.0%	5.26			HONEY-LIKE, LESS STICKY
NXHT12:DEBITTERING WATER	NXHT12R3	XAD-4 RESIN	EtOH 96°	COLUMN/SATURATION STUDY : 5BV+2 BV			1.68			HONEY-LIKE, LESS STICKY
NXHT12:DEBITTERING WATER	NXHT12SP2	RESIDUE	EtOH 96°	COLUMN/SATURATION STUDY : 5BV+2 BV			0.02			HONEY-LIKE, LESS STICKY
NXHT12:DEBITTERING WATER	NXHT12SP5	RESIDUE	EtOH 96°	COLUMN/SATURATION STUDY : 5BV+2 BV			0.08			HONEY-LIKE, LESS STICKY
NXHT12:DEBITTERING WATER	NXHT12SP8	RESIDUE	EtOH 96°	COLUMN/SATURATION STUDY : 5BV+2 BV			1.84			HONEY-LIKE, LESS STICKY
NXHT12:DEBITTERING WATER	NXHT12SP10	RESIDUE	EtOH 96°	COLUMN/SATURATION STUDY : 5BV+2 BV			2.2			HONEY-LIKE, LESS STICKY
RESIN TECHNOLOGY-DEBITTERING WATER										
NXHT12:DEBITTERING WATER	NXHT12IndExt	XAD-4 RESIN	EtOH 96°		0.85		9.98			HONEY-LIKE, LESS STICKY

Table 15. Evaluation of various olive processing by-products as sources of HT by green technological means

The main findings of the implemented comparative study can be summarized as follows:

- The olive paste (NXHT2) is a suitable material for supercritical CO_2 extraction, optimally with the addition of a small percentage of cellulose but not necessarily so. SFE proved to be efficient to extract HT, though recovery was not high, especially when there was no EtOH as a co-solvent (1.53% vs 4.92%).This led to an extract containing 0.32% OH-Ty. The recovery was even better when lyophilization of the raw material preceded the extraction (7.62%) and resulted to a lipophilic extract containing 0.05% HT as the yield was extremely enhanced due to the extraction of heavy lipophilic compounds. It was observed that, comparing to all the other methods, the extracts with SFE presented the more versatile profile in terms of polyphenols and some terpenes and lipids. The SFE extracts that were obtained at 100 and 200 bar were co-chromatographed in HPLC with some standard compounds from Pharmacognosy laboratory chemical library. This permitted to identify some of the compounds that are present, such as oleacein, oleocanthal, acetoxypinoresinol, aldehydic oleuropein aglycon, elenolic acid and its isomers etc.
- Maceration with boiling water had a very poor recovery while maceration in EtOH presented the best recovery among all the methods used (60%) and resulted to an extract containing 0.68% HT. Both procedures presented some filtration difficlulties as the nature of the material is pasty.
- PLE with EtOH had a recovery of 8.4%, though larger contact (3 cycles of 5 min. were performed) with the material would clearly increase significantly the recovery. The extract contained 0.21% HT.
- This was even more evident concerning subcritical water at 190 °C were same (short) extraction scheme resulted a recovery of 32.4%. The extract contained 0.30% HT.
- Concerning the use of Adsorptive Resins (ART) for the liquid RMs, this was proved to be very efficient in lab scale as well as in large scale, as XAD-4 resin was able to retain the olive polyphenols (especially HT) very well, provided

that for each RM a saturation study has been performed in lab-scale so that maximum feed and minimum EtOH quantity are defined. Extracts containing up to 11.41% of OH-Ty were obtained from the debittering water.

- The study –in lab scale- for the resin column saturation when TOPW was fed and optimal EtOH volume for the recovery of the adsorbed phenolic load revealed that 5bed volumes (BV) of TOPW was the maximum quantity that that could be fed to the resin before saturating its adsorption capacities. Regarding the desorption, 2 BV of EtOH were sufficient for the recovery of the phenol content (TOPWPF).
- Industrial implementation of ART for NXHT4 resulted to an extract relatively poorer in HT (1.23%).
- The majority of the extracts had an aspect of sticky paste. SFE extracts differentiated being more lipophilic (it contained triterpenes and lipids). NXHT12 (TOPW) extracts were honey-like concerning the color and viscosity, but less sticky. They present an odor of olives.
- It was calculated, according to the results, that from 1 ton of NXHT12 (TOPW) we should be able to obtain approx. 8.5kg of extract containing approx. 10% of HT (this can probably be optimized by adjusting the maturity of the debittering brine used and enhancing the NaCl removal). The resins 'capacity for the specific raw material is 5 bed volumes(BV) as it was observed, which means that in order to treat in one batch 1 ton of RM we need to have 200lt of XAD-4 resin and the recovery of the polyphenols is completed after rinsing with max. 2 BV of EtOH (400lt). Of course the resins, once properly regenerated, can be used many times without problem.

1.2.3.3b) Recovery of polyphenols from TOPW

As TOPW was proved to be the most abundant source of HT, the research proceeded with this material. For the establishment of the treatment, three parameters had to be evaluated:

- TOPWs produced from natural debittering of black (Kalamon variety) and green (Amfissis variety) olives were evaluated as HT sources, by applying a conventional extraction protocol.
- XAD-4 and XAD-7 macroporous resins were compared for their capacity to desorb HT as well as Tyrosol, in order o decide on the most suitable adsorbent.
- Liquid/liquid centrifugal extraction and separation parameters needed to be defined for the development of an alternative method of recovery of TOPW polyphenols.

For the by-product evaluation as best source, 150 mL of Kalamon-TOPW were dried in a rotary evaporator. The residue was redissolved in 200 mL of MeOH/ACE 50:50, filtered (from NaCl) and dried. The weight of the final dry residue was 1.66 g, and HPLC analysis showed that its HT content was 2.40%. Thus, the concentration of HT in the initial material was calculated to be 0.027% (w/v). TLC and RP-HPLC analysis showed that other phenolic compounds, especially tyrosol (TYR) and acteoside, were also present (Fig. 29). The Amfissis-TOPW was manipulated similarly to the Kalamon-TOPW for the estimation of the percentage of HT. 150 mL produced 9.66 g of dry extract containing 3.22% HT, meaning 0.207% of HT (w/v) (Fig. 29). In this case, also TYR was detected, but not acteoside. Consequently, the concentration of HT in Amfissis-TOPW was estimated to be approximately 10-fold higher than its concentration in Kalamon-TOPW, as it is summarized in Table 16.

In continuation, 250 mL of each TOPW were loaded on a glass column, filled with XAD-4 resin, previously activated by applying a sequential passing of EtOH and H2O. XAD-4 was selected as the most appropriate type of resin to successfully adsorb HT, according to manufacturers' specifications [http://www.advancedbiosciences.com/]. The resin was then washed with water to remove residual polysaccharides, lipids and salt. The phenolic fractions were collected by elution with 70 mL of EtOH, while the adsorption capacity of the resin and the point that HT would no longer be adsorbed was checked by TLC. The final dry extracts, Kalamon-TOPWPF and Amfissis-TOPWPF, produced after desorption with EtOH and evaporation of the solvent were 1.09 g (0.44% yield) and 2.82 g (1.13% yield), respectively. Analysis with a method developed on RP-HPLC revealed that the content of HT was 4.05% in the Kalamon-

TOPWPF and 10.10% in the Amfissis-TOPWPF. TYR and acteoside were further detected in the dry Kalamon-TOPWPF (Fig. 30) and TYR in the Amfissis-TOPWPF.

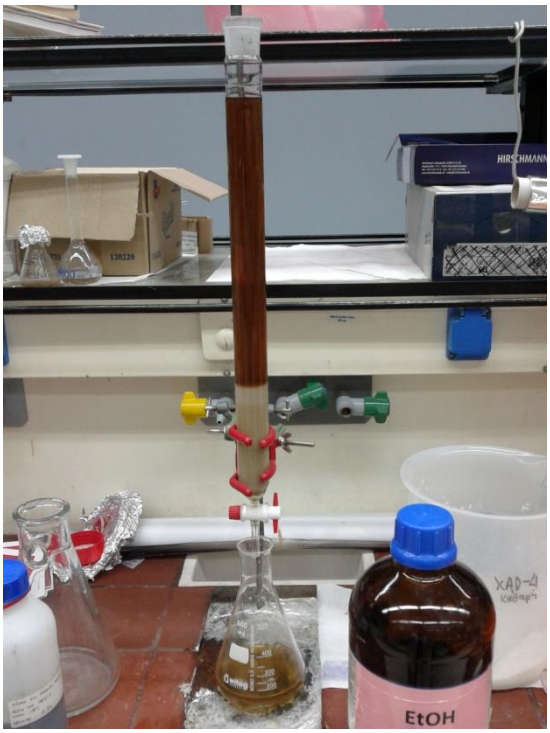


Figure 55. Kalamon TOPW treatment with XAD-4 resin in a column.

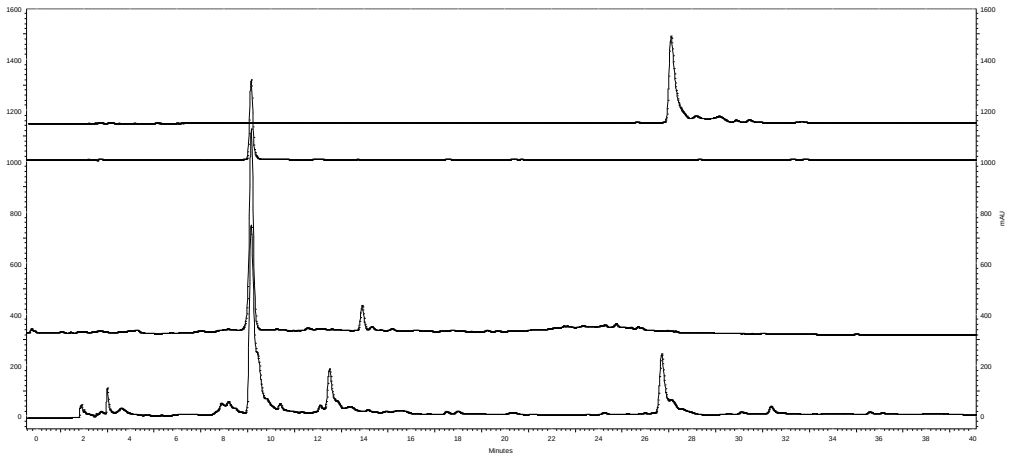


Figure 56. HPLC chromatograms (UV 280 nm) of (A) Kalamon-TOPW, (B) Amfissis-TOPW, (both from conventional extraction) (C) standard compound HT (D) standard compound acteoside.

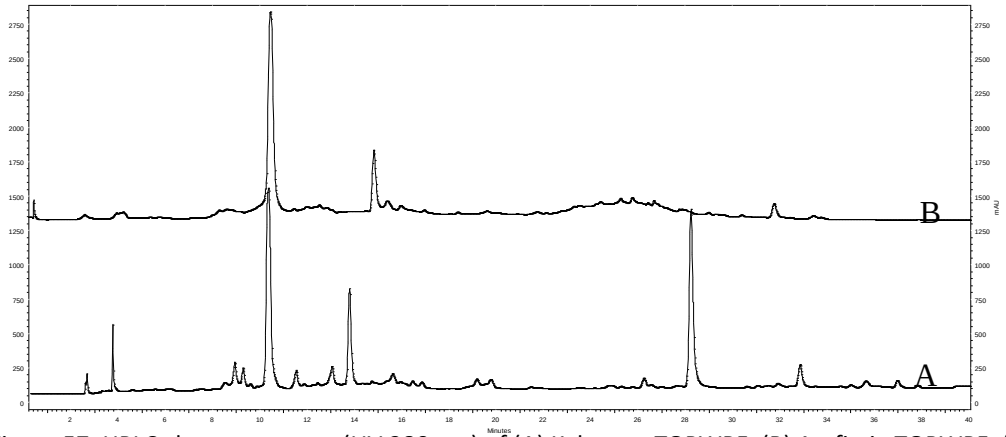


Figure 57. HPLC chromatograms (UV 280 nm) of (A) Kalamon-TOPWPF, (B) Amfissis-TOPWPF, (both after XAD4 resin treatment)

Furthermore, for the comparison of the two different resins, two columns were filled with 40 ml of XAD-4 and XAD-7 resins respectively, and 200ml of the same TOPW (Amfissis) was fed in both columns under the same flow rate (1-1,5 BV/hr). Consequently, the same quantity of EtOH (80ml) was fed to both columns in order to desorb the phenolic fraction that had been adsorbed upon the pores of the adsorbents. The extracts were evaporated to dryness and HT as well as Tyrosol (TYR) were quantified by HPLC-DAD, through calibration curves. The results lie in Table 16

	TABLE OLIVE PROCESSING WATER POLYPHENOL FRACTION (TOPWPF)	**HT**	**TYR**
XAD-4	6,28% w/w in TOPW (**7,951 Kg / 1000 L TOPW**)	10,1% w/w In TOPWPF	1,50 % w/w In TOPWPF
		$0,63_4$ % w/w In TOPW (**$803,01_5$ g / 1000 L TOPW**)	0,094 % w/w In TOPW (**119,05 g / 1000 L TOPW)**
XAD-7	$3,51_2$ % w/w In TOPW (**4,445 Kg / 1000 L In TOPW**)	15,6% w/w In TOPWPF	3,45 % w/w In TOPWPF
		$0,54_8$ % w/w In TOPW (**694,03 g / 1000 L TOPW**)	0,121 % w/w In TOPW (**153,24 g / 1000 L TOPW**)

Table 16. Comparative study between XAD-4 and XAD-7 polymeric adsorbents for the adsorption capacity for HT and TYR

The conclusions that were drawn from the above-mentioned study were:

- XAD-4 has a greater capacity of adsorbing TOPW polyphenols than XAD-7 (6.28 vs. 3.51%)
- XAD-4 treatment of TOPW leads to a greater recovery of HT than a XAD-7 treatment (0.63 vs. 0.55%)
- Nevertheless, the polyphenolic fraction of TOPW (TOPWPF) that is recovered by XAD-7 treatment is more rich in HT than with XAD-4 (15.6% vs. 10.1%)
- XAD-7 treatment of TOPW leads to a slightly greater recovery of TYR than a XAD-4 treatment (0.121 vs. 0.094%)
- The polyphenolic fraction of TOPW (TOPWPF) that is recovered by XAD-7 treatment is more rich in TYR than with XAD-4 (3.45% vs. 1.50%)
- The above mean that using XAD-4 leads to a greater quantity of TOPWPF, less rich though in HT and TYR particularly, but more rich in other phenols. Recovery of HT in overall is more effective with XAD-4, while recovery of TYR is slightly more effective with XAD-7.

Finally, for the countercurrent liquid/liquid extraction, the heavy phase is given and it is the TOPW. Thus, only the solvent(s) to form the second phase are required. Tests in test tubes with 2 different organic solvents have been performed to find the suitable extracting solvent system:

1) Ethyl acetate: slight emulsion has been remarked
2) Ethyl acetate / ethanol (9/1): total decantation takes 1 minute. The presence of 10 % of ethanol probably destroys the emulsion and stabilizes the system.

The TOPW is first filtered in a 6 µm filtration bag, in order to remove the low quantities of solid material that is contained.

Figure 58. 6µm filtration bag after TOPW filtration and TOPW after filtration

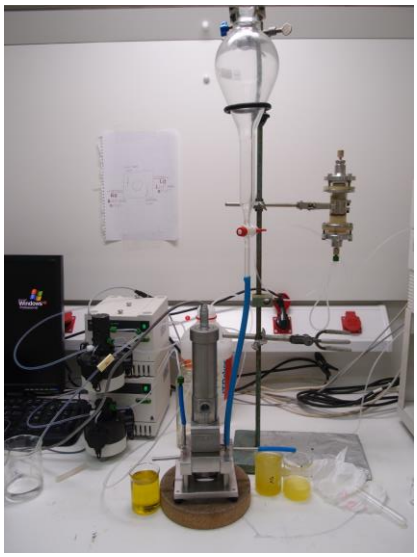


Figure 59. BXP012 liquid/liquid countercurrent extractor

Both heavy and light phase were fed into the BXP thanks to medium pressure membrane pumps. Flow rate could be easily adjusted by software. Heavy Phase is pumped in the BXP at flow rate 15 ml/min and 3600 rpm. Approximately 10ml is pumped and then flow rate is decreased to 5 ml/min. Secondly light phase is pumped at 5 ml/min, speed remains at 3600 rpm. The BXP is now "equilibrated". Several flow rate and rotation speed were tried as it can be seen in Table 17.

Trial	Rotation speed (rpm)	Heavy phase flow rate (ml/min)	Light phase flow rate (ml/min)	Results
1	3500	5	5	No pollution of both phases
2	5000	5	5	No pollution of both phases
3	7000	5	5	No pollution of both phases
4	10350	5	5	No pollution of both phases
5	10350	10	5	No pollution of both phases
6	10350	15	5	No pollution of both phases Sample n°1 has been taken

Table 17. Trials on BXP012 and parameters of the extractions

The TLC analysis of sample n°6 shows a very good extraction of polyphenols. The TOPW before and after extraction has been analyzed with TLC. This control showed that polyphenols are still present in the waste water after extraction on the BXP. Subsequently, HPLC-DAD analysis was performed in order to evaluate the content of the two compounds of interest in the final extract. The chromatographic analysis was performed with a Thermo Finnigan HPLC system (Thermo Finnigan, San Jose, CA) coupled with a Spectra System UV6000LP PDA detector. The separation was performed at 25 °C and the injection volume was 10 µL. The detection was performed at 280 nm and the column used was Supelco Analytical Discovery HS C18 (25 cm x 4.6 mm, i.d., 5.0 µm). As it can be seen in the chromatogram below, effectively, hydroxytyrosol and tyrosol were the two main compounds:

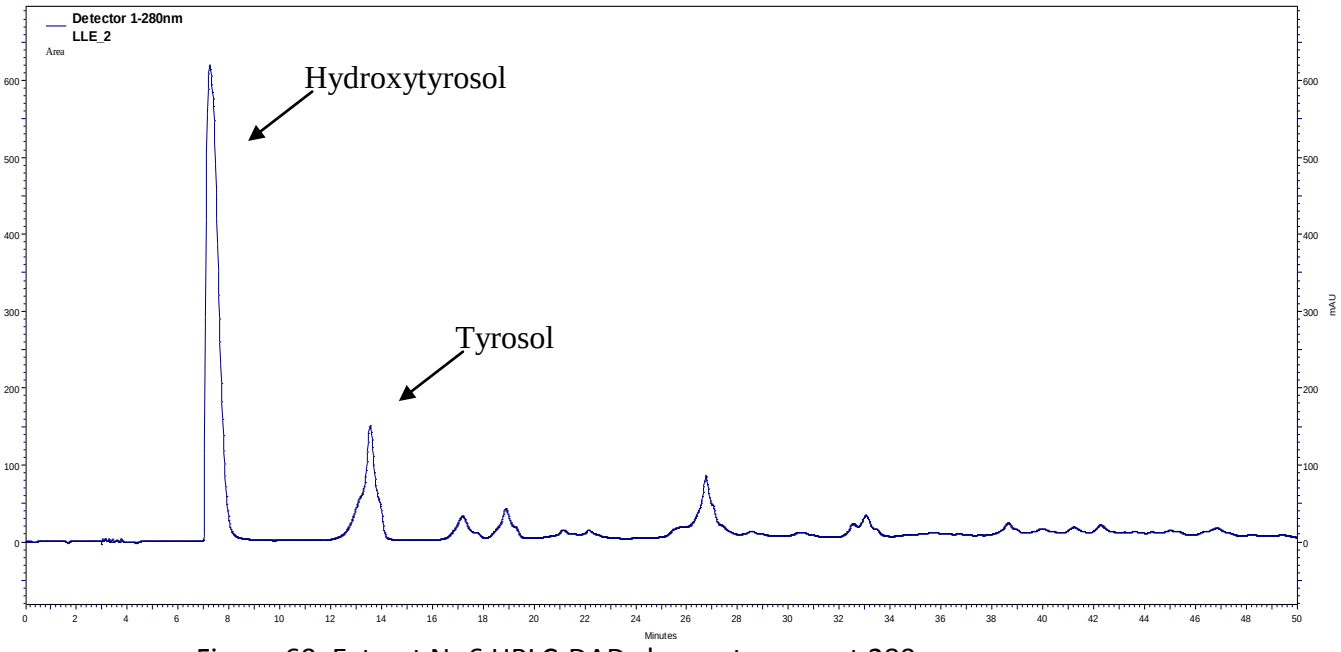
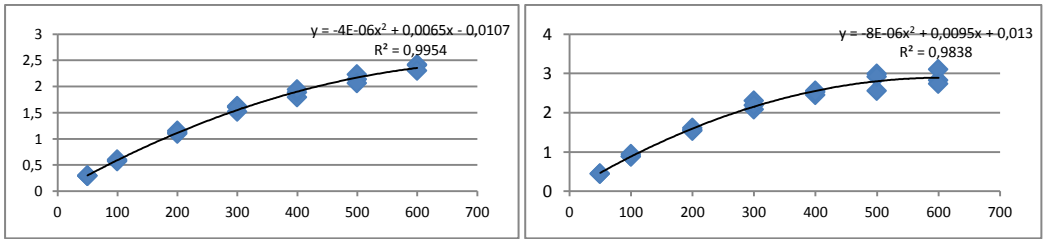


Figure 60. Extract No6 HPLC-DAD chromatogram at 280nm

Quantification of both components was performed through calibration curve construction. Below lie the calibration curves for each compound.



Thus, the final extract (extract No 6, maximum rotation speed) was analyzed and it was found to contain 12.4% hydroxytyrosol and 3.9% tyrosol. The liquid/liquid extraction with BXP012 results an enriched in hydroxytyrosol and tyrosol TOPW extract, comparable to the one recovered by the already established macroporous resin process. Also, filtration of the TOPW led to avoid the problem that the saltiness of this wastewater (containing 5-8% salt) caused by the plugging of the column due to crystallization of the salt. Finally, the continuous process of liquid/liquid extraction is advantageous comparing on the semi-batch process of the macroporous resins. Process duration can be significantly lowered by using liquid/liquid extraction. On the other hand, the unavoidable use of organic solvents, ethyl acetate in this case, is a disadvantage, especially for the nutritional supplement and food market. Thus, as not any significantly more important hydroxytyrosol content has been observed by using countercurrent liquid/liquid extraction, the resin process has been chosen to proceed with for its green advantage. More specifically, since the aim of the study has been the optimal HT recovery with view to an extract that is also rich in other olive phenols, XAD-4 was chosen as the adsorbent to proceed with. The process was transferred to pilot-scale for Amfissis-TOPW that was proven to be a richer source of HT and exhibited a more efficient enrichment with the XAD-4 resin treatment, the one that proved to be more efficient in polyphenol recovery from TOPW generally. Thus, 400 L of TOPW from Amfissis variety were fed to a XAD-4 resin column, while 150 L of EtOH were used for the recovery of the

phenolic compounds (Amfissis-TOPWPF). The solvent was evaporated under vacuum in a QVF glass 200 L evaporator and consequently in a Buchi 20 L rotary evaporator. This resulted to 1.34 kg of dry extract (0.34% yield), relatively lower than the yield observed at the lab scale. The extract was analyzed with RP-HPLC and its content in HT was 9.98%.

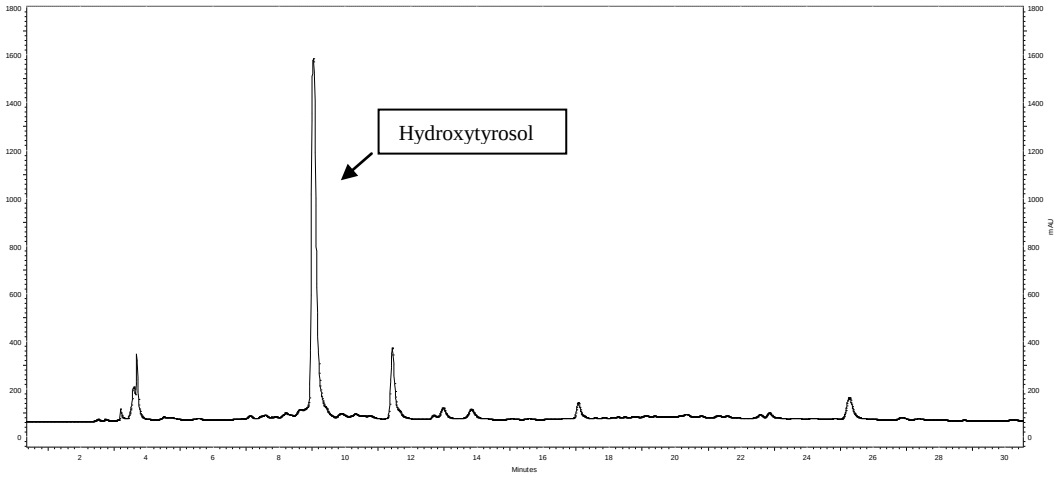


Figure 61. HPLC chromatogram (UV 280 nm) of Amfissis-TOPWPPF after XAD-4 resin treatment at pilot scale

1.2.3.3c) Purification of hydroxytyrosol

In order to isolate pure HT, the Amfissis-TOPWPF was submitted to CCC. The use of CCC was considered as the most effective, as this technique allows a total recovery of the sample, and so could lead to improved yields compared to other procedures. This was initially performed in a FCPC 1 L rotor instrument (preparative scale) and afterwards the method was scaled up to an SCCC 12.5 L instrument (pilot scale). Ten biphasic systems were tested and HPLC chromatograms showed that only TYR could co-elute with HT. Based on the Kd of HT and the ratio (α) of the Kd of HT and TYR, c-Hex/EtOAc/EtOH/H2O (1:9:2:8, v/v/v/v) was selected as the most appropriate solvent system.

SOLVENT SYSTEM	Kd HT	Kd TYR
c-Hex/EtOAc/EtOH/H2O 9:1:2:8	395.5	48.4
c-Hex/EtOAc/EtOH/H2O 8:2:2:8	65.8	11.6
c-Hex/EtOAc/EtOH/H2O 7:3:2:8	28.6	6.2
c-Hex/EtOAc/EtOH/H2O 6:4:2:8	11.0	2.6
c-Hex/EtOAc/EtOH/H2O 5:5:2:8	6.3	1.8
c-Hex/EtOAc/EtOH/H2O 4:6:2:8	3.5	1.1
c-Hex/EtOAc/EtOH/H2O 3:7:2:8	2.2	0.8
c-Hex/EtOAc/EtOH/H2O 2:8:2:8	1.5	0.6
c-Hex/EtOAc/EtOH/H2O 1:9:2:8	1.0	0.4
EtOAc/EtOH/H2O 10:2:8	0.7	0.4

Table 18. Kd for HT and TYR in 10 biphasic solvent systems

Initially, 6.0 g of Amfissis-TOPWPF were initially injected to the CCC. The HT purity was estimated by HPLC to be >95%.

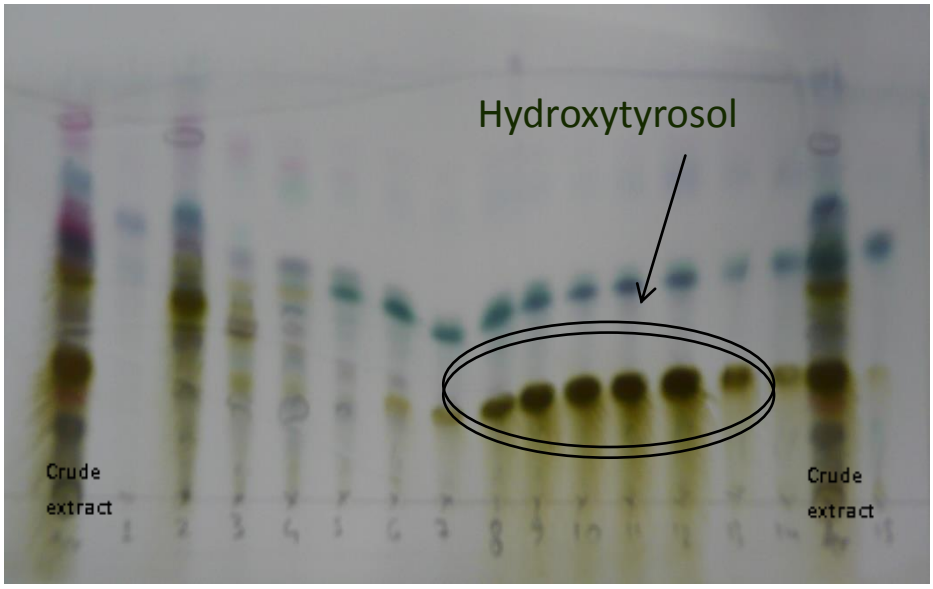


Figure 62. TLC of the various prep CPC fractions and the initial extract (TOPW Amfissis after XAD-4 treatment)

Further on, a pilot scale treatment was developed in order to check its up-scalability. In order to optimize productivity of compound to be purified on the CPC12,5L. different quantities of raw extract were injected. Table 19 summarizes general parameters and Table 20 is a resume of all injections done.

SCPC	Solvent System	Rotation speed	Flow rate	UV Detection
12,5L	Cyclohexane / Ethyl acetate / Ethanol / Water (2/8/1/9, v/v/v/v)	1000 rpm	200 ml/mn	280nm and 254nm

Table 19 :General CPC Parameters

Run N°	Injected Mass	Solvent Volume (for solubilisation) LP: Lower phase UP: Upper phase	Injected Total Volume	Running time
1	200 g	230ml LP + 20 ml UP	370 ml	155 mn
2	400 g	200ml LP	550 ml	158 mn
3	450 g	300ml LP	770ml	171 mn
4	500 g	300ml LP	750ml	133 mn
5	430 g	240ml LP	600ml	145mn
6	400 g	200ml LP	600ml	150 mn

Table 20 : Resume of all CPC12,5 injections

Figure 63. CCC with pilot column (Armen®, 2x6,25 lt) in ARMEN SA, Vannes, France

Figure 64. Solvent system preparation for CCC with pilot column (Armen®, 2x6,25 lt) in ARMEN SA, Vannes, France

During trials 1-5, an effort to optimize the quantity that could be injected and maintain good resolution has been made. It was concluded from the quality of separation according to UV-detection and TLC that a maximum quantity of 400g of TOPWPF Amfissis could be injected in the SCPC 12,5lt in order to maintain high resolution. This led to trial 6 that resulted 37.8g of HT. The purification lasted approximately 4 hours including extrusion and requires ~22L of organic solvents + 28L of demineralized water with a cost of ~75-80 euros. HT was isolated in a pure form, while the yield was considerably improved, as can be seen in Table 17. Purity of the isolated compound was also confirmed by quantitative HPLC and 1H-NMR (Figures 67, 68 .)

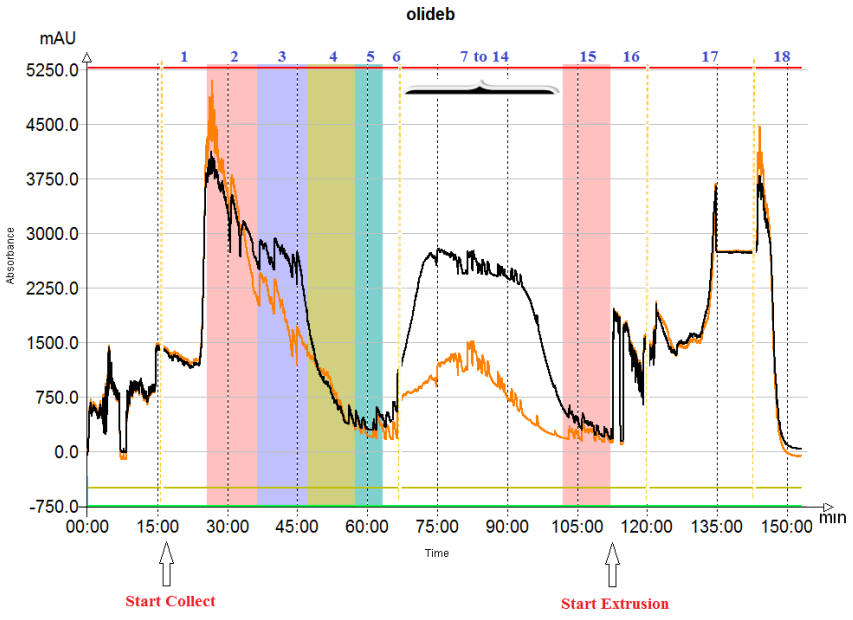
For the sample preparation, 200g Crude extract was solubilized in 230 lower phase and 20ml of upper phase of system solvent let be a concentration of 800g/L. The CPC Conditions are summarized below :

- Solvent system : cyclohexane/ethyl acetate/Ethanol/Water(2/8/1/9).
- Injection volume : 220 ml lower phase and 30ml of upper phase; Rotation Speed : 1000 rpm; Flow rate : 200 mL/mn;
- Stationary phase retention: 74%; Run time : 155 mn

- Loading of lower stationary phase in ASC mode, 500 rpm, 800 ml/mn on CPC 12.5L
- Equilibrium with upper mobile in ASC mode, 1000 rpm, 200 ml/min
- After retention, sample injection via injection pump at 50 ml/mn
- Elution with upper mobile phase, 1000 rpm, 200 ml/mn
- Beginning of extrusion after elution, 1000 rpm, 500 ml/mn
- Detection at 254 et 280 nm
- Collection according to chromatogram evolution.
- Retention volume : Vm = 3.2L, Rt = 74%
- Equilibrium Pressure: 30bars
- Collection, Table 21: (in red: fractions with purify compound, in green: extrusion fractions)

Fractions	1	2	3	4	5	6	7	8	9
Time	15mn 30	26mn 30	37mn 30	47mn 05	57mn 20	63mn 15	66mn 30	68mn20	72mn 35
Fractions	10	11	12	13	14	15	16	17	18
Time	77mn	82mn	87mn	92mn	97mn	102mn	112mn	120mn	143mn 25

Table 21. Fraction collection during run 6. See respective chromatogram and TLC below.



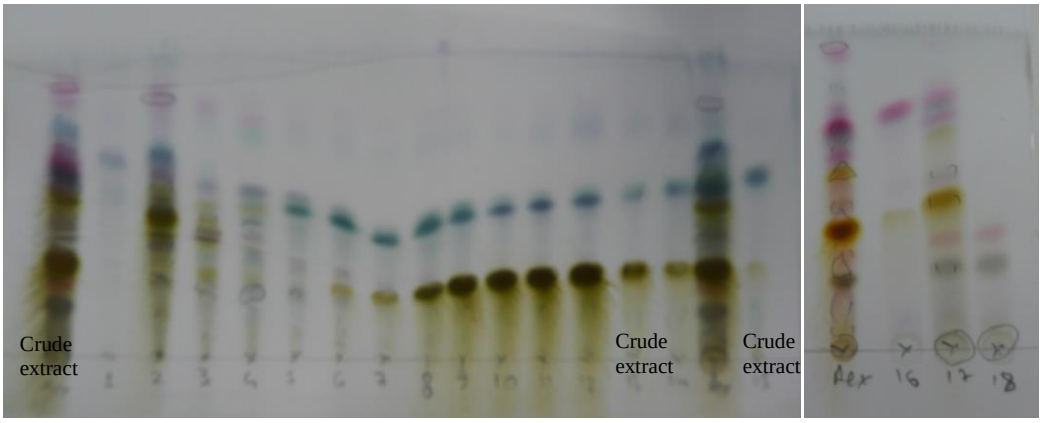


Figure 65 : CPC chromatogram and TLC Analysis of run N°6

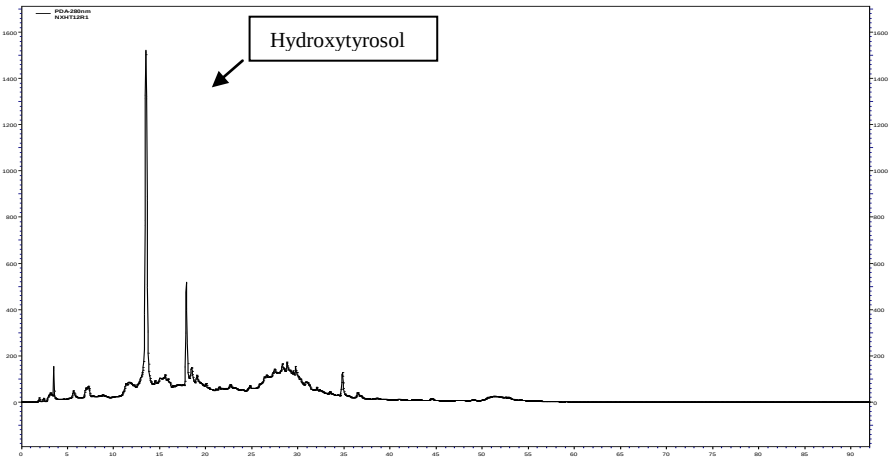


Figure 66 . HPLC-DAD chromatogram of HT isolated from Amfissis-TOPWPPF resin extract (pilot CCC)

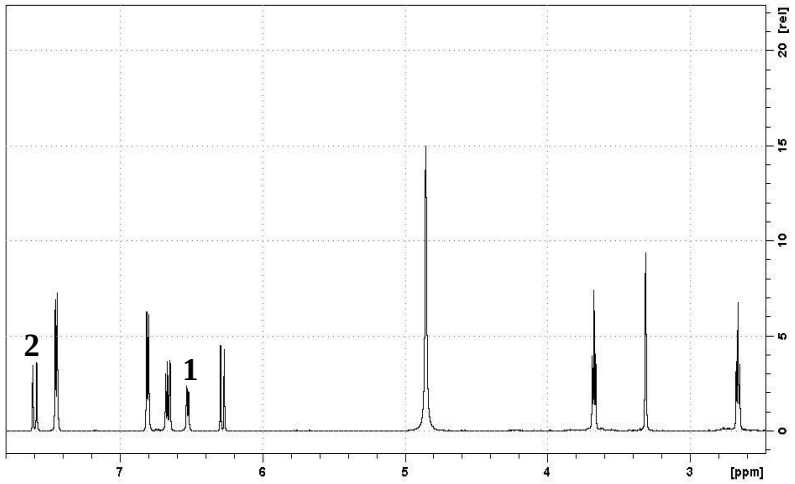


Figure 67. 1H-NMR spectrum of HT isolated from Amfissis-TOPWPF resin extract (preparative CCC) and p-coumaric acid: 1 = the dd from H-6 of HT at 6.52 ppm used for quantitation and 2 = the d from H-7 of p-coumaric acid at 7.60 ppm.

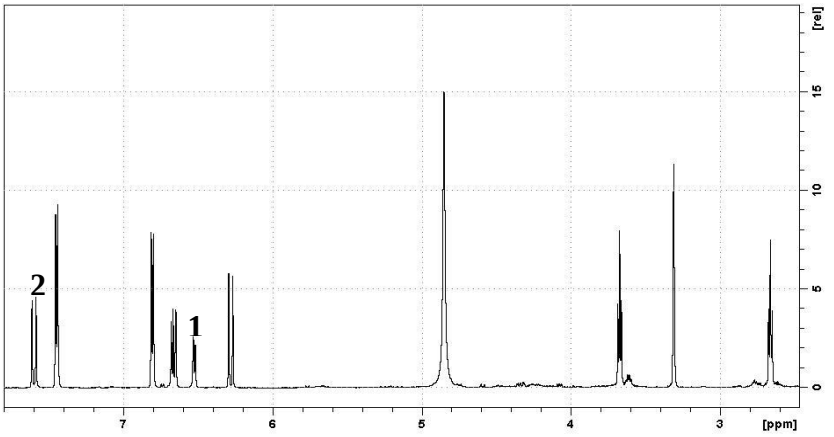


Figure 68. 1H-NMR spectrum of HT isolated from Amfissis-TOPWPPF resin extract (pilot CCC) and p-coumaric acid: 1 = the dd from H-6 of HT at 6.52 ppm used for quantitation and 2 = the d from H-7 of p-coumaric acid at 7.60 ppm

	Initial material (TOPW)	
	Kalamon	**Amfissis**
extract (g)	1.66	9.66
%HT content (w/v)	0.027	0.207

Table 22. Hydroxytyrosol content of the two initial materials (TOPW Amfissis and Kalamon

	Phenolic fraction (TOPWPF) lab scale resin		Phenolic fraction (TOPWPF) pilot scale resin	CCC preparative scale	CCC pilot scale
	Kalamon	Amfissis	Amfissis	Amfissis	Amfissis
extract (g)	1.09	2.82	1340	0.432	37.8
% yield (w/v)	0.44	1.13	0.34	7.20	9.45%
% HT content (w/w)	4.05	10.10	9.98	pure compound	pure compound

Table 23. Yield and Hydroxytyrosol content/purity for the various extracts and purified fractions

1.2.3.4) Concluding remarks

The aim of the present study was to trace the olive processing by-product which is more rich in HT and to develop an effective (both qualitatively and quantitatively) procedure designated for the extraction and isolation of HT from this. The debittering process causes the fruit to lose a major part of its phenolic content, due to the diffusion of these compounds from olives to brine, making the latter a good source of polyphenols and their recovery of major interest. The present study established a feasible procedure for producing extracts rich in HT. It combines two highly selective chromatographic techniques for the separation and isolation of HT, avoiding tedious purification methods. The developed methodology involves the production of an extract, rich in phenolic compounds, eluting from XAD-4 resin and the isolation of HT with CCC. The phenolic fraction deriving from the first step (Amfissis-TOPWPF) was obtained from the XAD-4 resin effluent, without any pretreatment and contained 10.10% HT. The developed CCC methodology allowed the isolation of >95% pure HT directly from the Amfissis-TOPW. It should be emphasized that the complexity of the process is small, as is the solvent consumption and the overall time needed. Moreover, it allows the recovery of HT with high efficiency, low cost, facility in scale production, uses chemical-free methods, features that make this process economically feasible and possible to be

applied also in pilot scale with similar results. The positive results prove this low-cost procedure could be an alternative to the conventional extraction method for obtaining antioxidant phenolic extracts and pure compounds. Its effectiveness could be also applied to other constituents of TOPW, like TYR and acteoside. The proposed process could be considered as a new approach combining environmental protection and production of high added value compounds useful in the nutraceutical, pharmaceutical and cosmetics industry.

1.3) Investigation for the richest source of squalene (SQ) among various olive kernel oil reffineries' by products. Squalene enrichment by means of supercritical fluid extraction. Development of a single-step isolation of squalene by non-aqueous hydrostatic countercurrent chromatography (CPC).

1.3.1) Introduction

Olive oil and olive kernel oil deodorizer distillates (OODD) are a by-product of the olive (kernel) oil refining process, representing approximately 1,5% of the crude olive oil. It is normally disposed as a waste though it contains high amounts of free fatty acids (FFA) and unsaponifiables. Among these, the most abundant is squalene (SQ), an aliphatic triterpene. OODD occur in large quantities in several facilities around the Mediterranean basin. The refining process for the olive kernel oil that has a relatively high acidity consists of several steps, mainly neutralization, discoloration and deodorization. During deodorization and in facilities that use high vacuum (0,3mbar) and temperature (min. 230˚C) distillation of SQ and other unsaponifiables occurs along with this of the FFAs.

Many researchers have worked on olive oil deodorizer distillates in the past, but very few, if any, in olive kernel oil similar by-products. Olive oil, according to literature reports, contains 0.08-1.20 % SQ [Auffray et al, 2009 and Samanieg-Sanchez et al., 2010] while 60-70 % of the unsaponifiable fraction of olive oil is consisted of SQ [Murkovic et al., 2004]. Certain studies have been conducted for the separation and purification of SQ from olive oil deodorizer distillates, most often implementing countercurrent supercritical fluid extraction (CCSFE). Thus, Akgun N.A. has reported the recovery of SQ from olive oil deodorizer distillates in a purity of 73.74% in two steps, by applying esterification in supercritical methanol and subsequently CCSFE optimized via response surface methodology [Akgun, 2011]. Vazquez et al. reported the recovery of SQ from an olive oil deodorizer distillate containing 52% in a CCSFE pilot plant with 15 theoretical stages, achieving a purity of 89.4% [Vazquez et al., 2007]. Bondioli et al. reported the recovery of SQ from olive oil deodorizer distillates by CCSFE, implementing a first step of saponification. The purity of SQ was 83.7% [Bondioli et al., 1993]. Purification of SQ in a purity of 96%.by means of high-speed countercurrent chromatography (HSCCC) from the microalga

Traystochytrium ATCC 26185 has been reported by Lu et al [Hai et al., 2003]. This work has been performed in analytical scale and concerned the organic extract of the microalgae. Similarly, the isolation of 75% pure SQ from olive oil by means of HSCCC has been reported by Schroder and Wetter [Schroder et al., 2012]. This work has also been performed in analytical scale, while it concerned a noble product as is olive oil.

Despite the fact that alternative sources of SQ have been described in by-products of a relatively large industry as is olive oil refining industry, no olive kernel oil by-product has been studied, neither any preparative chromatographic purification from these has been described in the literature. At the same time, shark liver oil remains up-to-date the main source of SQ. One of the main advantages of countercurrent chromatography (CCC) is the increased load capacity: contrary to solid-liquid chromatography which requires a small volume of injection to ensure a good resolution, large quantities of sample can be introduced into the system. Furthermore, the absence of solid phase avoids irreversible adsorption and so allows a total recovery of the injected sample.

1.3.2) Aim of the study

From the laboratory and industrial experience, it was known that olive (kernel) oil refining by-products contain SQ. This is due to the removal of SQ along with colour, odours and acids from the oil during the process. Thus, the aim of this experimental project was dual since the beginning:

- Mapping of the refining process, complete sampling of all by-products as well as start, intermediate and end products and analysis for the quantitative assessment of SQ in all these.
- SQ enrichment and purification from the richest source (by-product) by means of green and viable processes, such as SFE and CPC.

1.3.3) Materials and methods

1.3.3a) Chromatographic Apparatus

Preparative hydrostatic CCC separations were carried out on a Kromaton FCPC® instrument equipped with a rotor of 1000 mL. OODD was injected into the system

through a 50 mL loop, and fractions were collected with a Büchi B-684 fraction collector.

1.3.3b) Reagents and materials

Hydrostatic CCC separations were performed using organic solvents of analytical grade (Sigma-Aldrich). Acetonitrile, used for UHPLC-DAD were of HPLC grade and purchased from Merck. Standard SQ was purchased from Sigma-Aldrich. OODD was kindly provided by olive oil refining industry ELSAP. (Nafplio, Greece). In all experimental protocols described below, OODD –which has an oily and a semi-solid phase- is primarily agitated in order to provide a homogeneous mixture during the sample preparation.

1.3.3c) Supercritical fluid extraction

SFE was performed in Separex 1-2 semi-pilot apparatus using SC-CO2 with or without EtOH as co-solvent. Both the 1 and 2 liter extractors were utilized, while as auxiliary for the extraction/fractionation, cellulose and/or silica gel were added to the initial material inside the extraction baskets, depending on the experimental strategy.

1.3.3d) Hydrostatic CCC separation procedure

Preparative separations were carried out on a Kromaton (Angers, France) FCPC® instrument equipped with a rotor of 1000 mL. The solvent was pumped through the system with a preparative pump LabAlliance, and the sample was injected via a 30 mL sample loop.

1.3.3e) UHPLC-DAD analysis

Analyses were performed on a Waters Acquity UHPLC system equipped with a photodiode array detector. Before analysis, a Solid Phase Extraction (SPE) protocol was developed for the elimination of the most polar compounds in OODD such as carotenoids and the majority of FFA. This was applied in a standard mode before OODD analysis and regarded the deposition of 150mg of OODD in an SPE silica cartridge (Silica gel for column chrom, ultra pure, 40-60μm, 60A) and its elution with 8 ml of heptane, which proved to provide full recovery of SQ while it left behind

more polar compounds. Finally, separations were carried out on an Acquity UPLC BEH C18 column (50 × 2.1 mm i.d., 1.7 µm). The injection volume was 1 µL. Elution was performed at 40°C, at a flow rate of 600 µL/min with the following isocratic solvent system: Acetonitrile 100%. Sample quantifications were made by constructing a calibration curve with different concentrations of standard SQ. R2 of the curve was 0.9924.

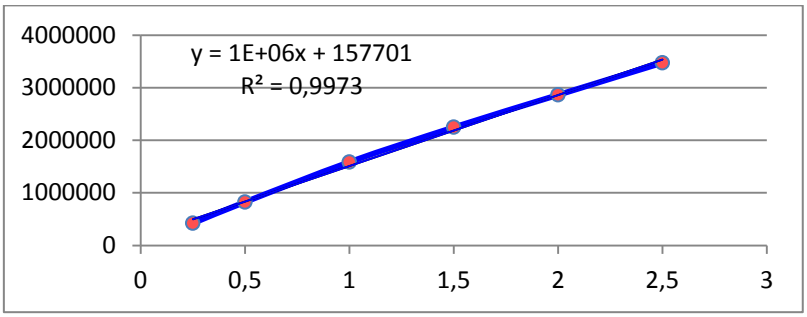


Figure 69. Calibration curve for Squalene in UPLC

1.3.3f) TLC analysis

OOD partition and CCC fractionation were first monitored by TLC, using the following elution system: Dichloromethane/Heptane (80:20 v/v). TLC plates (Silica gel 60 F254, Merck) were used and then sprayed with sulfuric vanillin.

1.3.3g) GC-MS analysis

OODD was firstly analyzed by GC-MS in order to identify the major compounds. Prior to analysis, an identical SPE protocol to the one implied for UHPLC was executed in order to pre-concentrate in SQ and to have a simpler mixture for analysis. Analysis was performed on a Finnigan DSQ apparatus, using a DB-5 column and He as gas, with a splitless method and 2µl injection. At the beginning of the method, temperature was. at 60˚C, then it was gradually raised 10˚C/min. for 24 min. up to 300˚C and it remained for 10 min. at 300˚C. Total analysis time is 33'. The eluting compounds were analyzed by Electron Impact and the relevant spectra were compared to those in WILEY 275L library for the identification of the compounds. SQ elution time is between 24.7 and 24.9 min. A calibration curve is constructed with

various concentration SQ solutions (25,50,100,200,400,600,800 and 1000 mg/ml) as seen in figure 66 below:

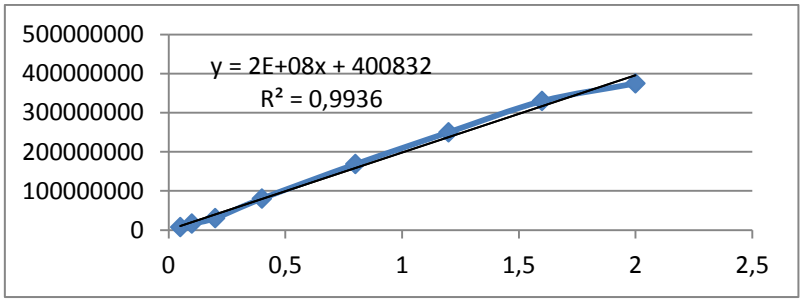


Figure 70. Calibration curve for Squalene in GC-MS

1.3.4) Mapping of the refining procedure and sampling

The aim of the mapping of the refining procedure is to collect without exception the initial and final products, by-products and intermediate products. This is ensured by the mapping of the process and sampling, in order to study the presence of squalene. Not only by-products are of interest but also the initial / final and intermediate products in order to study the variation of squalene in the process of refining olive (kernel) oil. This process basically has to do with converting non- edible oil in edible. The process involves a three-stage treatment:

- removal of margarines and neutralization of acidity
- decolorization and
- deodorization

Before the beginning of the process, the olive paste coming from the olive oil factory is initially placed in a desiccator in order to reduce the humidity of 50 %, which is initially in 10 % (two steps). The resulting material is subsequently extracted by hexane and separation of the liquid (micella) from the solid residues occurs. The latter are used as fuel to produce useful energy for the operation of the plant. Micella is then distilled to remove the organic solvent, which is recycled, and the oil is led to the refinery to be submitted to refining. The first stage of this is carried out for the removal of margarines and the neutralization of free fatty acids from the oil. This is done with the use of caustic soda, which has the capacity to neutralize the

free fatty acids, that directly affect the quality of the oil, creating soaps. After this process, neutral oil is obtained which is then decolorized and deodorized. Decolorization and deodorization are the most important stages in the process of refining olive kernel oil. In the flow chart below (Figure 67) are outlined the various stages of the refining procedure and points of sample collection are marked with color:

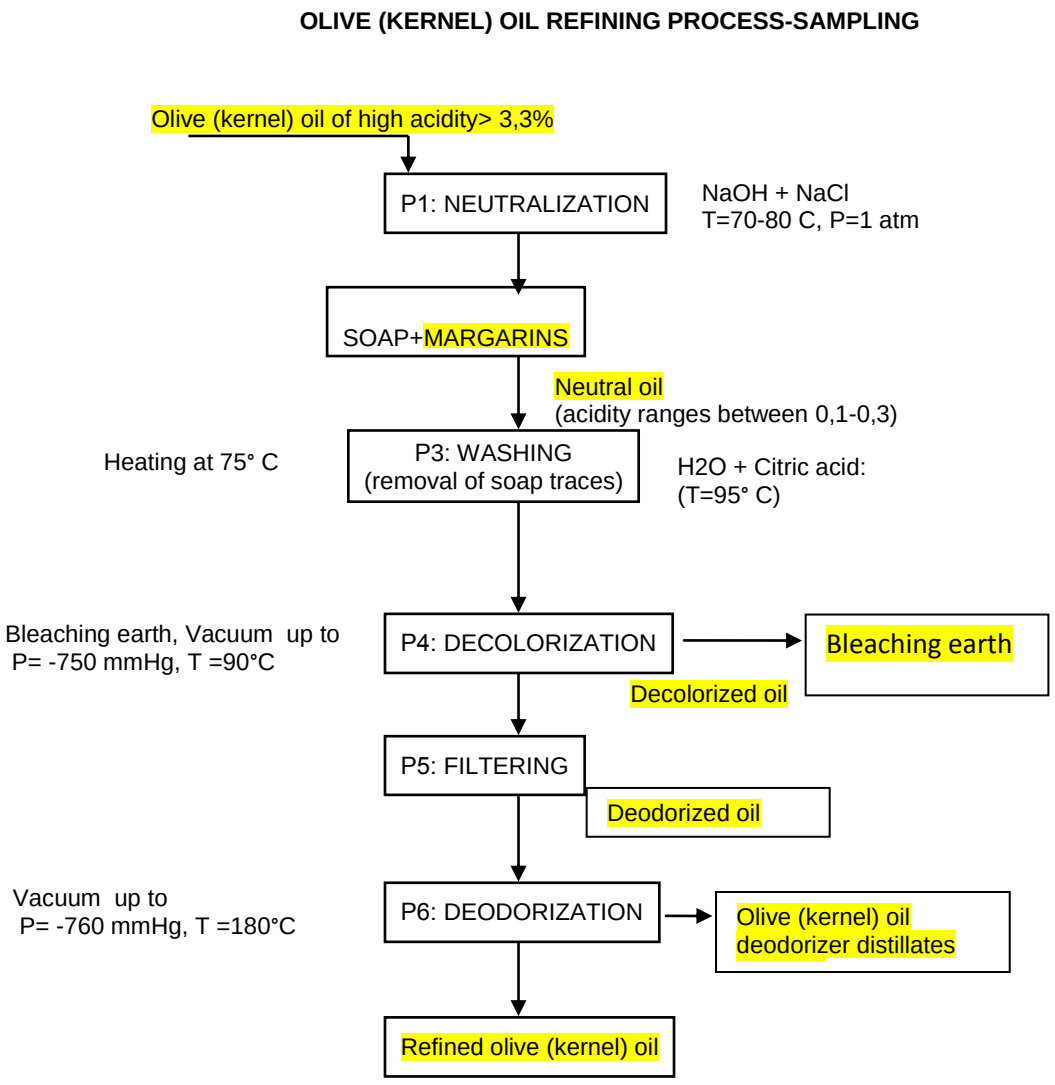


Figure 71. Olive kernel oil process flow chart and sampling spots (yellow)

In the codification, it is recorded in the different samples (3 to 5 for each type of product or by-product) the lot, also the physicochemical form and coded as indicated in Table 24:

Description	Form	Codification
Olive kernel oil unprocessed	Oily	5S3AP 1-3
Neutralization paste	Paste	5S3PEP 1-3
Olive kernel oil neutralized	Oily	5S3EXP 1-3
De-margarinization paste	Paste	5S3PAP 1-3
Olive kernel oil decolorized	Oily	5S3PAX 1-3
Bleaching earth	Viscous Paste	5S3PAG 1-3
Free Fatty Acids from deodorization (oily phase)	Oily	5S3ELOL 1-3
Free Fatty Acids from deodorization (solid phase: paste)	Paste	5S3ELOP 1-3
Refined olive kernel oil	Oily	5S3PR 1-3
Olive kernel oil unprocessed	Oily	1S3AP 1-3
Neutralization paste	Paste	1S3PEP 1-3
De-margarinization paste	Paste	1S3PAP 1-3
Free Fatty Acids from deodorization (oily phase)	Oily	1S3ELOL 1-3
Free Fatty Acids from deodorization (solid phase: paste)	Paste	1S3ELOP 1-3
Refined olive kernel oil	Oily	1S3PR(EP) 1-3
Olive kernel oil unprocessed	Oily	2S3AP 1-3
Neutralization paste	Paste	2S3PEP 1-3
De-margarinization paste	Paste	2S3PAP 1-3
Free Fatty Acids from deodorization (oily phase)	Oily	2S3ELOL 1-3
Free Fatty Acids from deodorization (solid phase: paste)	Paste	2S3ELOP 1-3
Refined olive kernel oil	Oily	2S3PR(EP) 1-3
Olive kernel oil unprocessed	Oily	4S3AP 1-3
Neutralization paste	Paste	4S3PEP 1-3
De-margarinization paste	Paste	4S3PAP 1-3
Refined olive kernel oil	Oily	4S3PR(EP) 1-3
Olive oil unprocessed	Oily	ES3AE 1-5
Neutralized olive oil	Oily	ES3EXE 1-5
Olive oil Neutralization paste	Paste	ES3PEE 1-5
Decolorized olive oil	Oily	ES3EAX 1-5
Bleaching earth (from olive oil bleaching)	Viscous Paste	ES3PAG 1-5
Refined olive oil	Oily	ES3EP(PR) 1-5
Free Fatty Acids from olive oil deodorization (oily phase)	Oily	ES3ELOL 1-5
Free Fatty Acids from olive oil deodorization (solid phase: paste)	Paste	ES3ELOP 1-5

Table 24. Samples from olive (kernel) oil refining process sampling for the tracing of SQ as well as its variation recording in the various products.

Figure 72. Samples from olive (kernel) oil reffinery

The samples were analyzed according to the protocols described previously (see materials and methods) and the results are presented in Table 25 below:

Sample	Area (y)	Squalene quantity injected (µg)	Starting product quantity to treat (mg)	Mixture quantity extracted (mg)	Substance solution prepared %o (mg/ml)	Solution quantity injected (µl)	Mixture quantity injected (µg)	Squalene quantity in extracted mixture (µg)	Squalene quantity in starting product (%)	Average
5S3AP1	7153589	0,0337638	150	31,9	3	2	6	179,51079	0,1196739	*0,100507*
5S3AP2	6156259	0,0287771	150	30	3	2	6	143,88568	0,0959238	
5S3AP3	5031444	0,0231531	150	33,4	3	2	6	128,88537	0,0859236	
5S3PEP1	0	-0,0020042	5000	83,4	3	2	6	-27,857824	-0,0005572	*0,000481*
5S3PEP2	636316	0,0011774	5000	134,9	3	2	6	26,472326	0,0005294	
5S3PEP3	1007276	0,0030322	5000	145,6	3	2	6	73,581872	0,0014716	
5S3EXP1	3682456	0,0164081	150	63,7	3	2	6	174,19954	0,116133	*0,097646*
5S3EXP2	4603610	0,0210139	150	52,7	3	2	6	184,572	0,123048	
5S3EXP3	1342103	0,0047064	150	102,8	3	2	6	80,635549	0,053757	
5S3PAP1	1395898	0,0049753	5000	212,3	3	2	6	176,04376	0,0035209	*0,003339*
5S3PAP2	596489	0,0009783	5000	359,8	3	2	6	58,664491	0,0011733	
5S3PAP3	1084052	0,0034161	5000	467,4	3	2	6	266,11419	0,0053223	
5S3PAX1	21236283	0,1041773	150	21,5	3	2	6	373,30183	0,2488679	*0,273705*
5S3PAX2	8632972	0,0411607	150	34,7	3	2	6	238,04605	0,1586974	
5S3PAX3	24491185	0,1204518	150	30,9	3	2	6	620,32659	0,4135511	

5S3PAG1	263668	-0,0006858	5000	125	3	2	6	-14,287917	-0,0002858	*-0,00025*
5S3PAG2	290950	-0,0005494	5000	173,94	3	2	6	-15,927396	-0,0003185	
5S3PAG3	357906	-0,0002146	5000	195,97	3	2	6	-7,0101735	-0,0001402	
5S3ELOL1	266019284	1,3280923	150	31	3	2	6	6861,81	4,57454	*12,68457*
5S3ELOL2	449949479	2,2477432	150	80,1	3	2	6	30007,372	20,004915	
5S3ELOL3	371251988	1,8542558	150	65,4	3	2	6	20211,388	13,474259	
5S3ELOP1	211288774	1,0544397	5000	3591,4	3	2	6	631152,46	12,623049	*13,27138*
5S3ELOP2	173711731	0,8665545	5000	5267	3	2	6	760690,42	15,213808	
5S3ELOP3	139552801	0,6957598	5000	5164,4	3	2	6	598863,69	11,977274	
5S3PR1	1719951	0,0065956	150	16	3	2	6	17,588253	0,0117255	*0,008071*
5S3PR2	1651883	0,0062553	150	27,1	3	2	6	28,252902	0,0188353	
5S3PR3	0	-0,0020042	150	28,5	3	2	6	-9,51976	-0,0063465	
ES3AE1	384776145	1,9218766	151,6	1,6	1,5	4	6	512,50042	0,338061	*0,310785*
ES3AE2	134595201	0,6709718	150,3	1,2	1,5	4	6	134,19437	0,0892843	
ES3AE3	420570119	2,1008464	152,4	1,7	1,5	4	6	595,23982	0,3905773	
ES3AE4	250646470	1,2512282	149,4	1,8	1,5	4	6	375,36846	0,2512506	
ES3AE5	314519395	1,5705928	151,2	2,8	3	2	6	732,94331	0,4847509	
ES3EXE1	293535151	1,4656716	151,8	1,9	1,5	4	6	464,12934	0,3057506	*0,355305*
ES3EXE2	224242242	1,1192071	150	2,1	3	2	6	391,72247	0,2611483	
ES3EXE3	301952774	1,5077597	149,4	2,4	3	2	6	603,10388	0,403684	
ES3EXE4	141082132	0,7034065	151,3	3,6	3	2	6	422,0439	0,2789451	
ES3EXE5	351730735	1,7566495	150	2,7	3	2	6	790,49228	0,5269949	
ES3PEE1	638477	0,0011882	5000	12,3	3	2	6	2,4358613	4,87E-05	*0,012933*
ES3PEE2	0	-0,0020042	5000	10,5	3	2	6	-3,50728	-7,01E-05	
ES3PEE3	274487778	1,3704347	5000	13	3	2	6	2969,2752	0,0593855	
ES3PEE4	4875237	0,022372	5000	70,7	3	2	6	263,61703	0,0052723	
ES3PEE5	622733	0,0011095	5000	7,2	3	2	6	1,331406	2,66E-05	
ES3EAX1	193025462	0,9631232	151,2	1,3	1,5	4	6	208,67668	0,1380137	*0,338244*
ES3EAX2	231116896	1,1535803	149,3	2,3	3	2	6	442,20579	0,2961861	
ES3EAX3	634753055	3,1717611	150,9	1,2	1,5	4	6	634,35222	0,4203792	
ES3EAX4	322260502	1,6092984	152,5	2,3	3	2	6	616,8977	0,4045231	
ES3EAX5	292896401	1,4624778	152,3	2,7	3	2	6	658,11503	0,4321176	
ES3PAG1	230506316	1,1505274	5000	21,7	3	2	6	4161,0742	0,0832215	*0,050944*
ES3PAG2	133286560	0,6644286	5100	58,4	3	2	6	6467,1054	0,126806	
ES3PAG3	27289759	0,1344446	5100	32	3	2	6	717,03805	0,0140596	
ES3PAG4	185951509	0,9277534	5100	10,7	3	2	6	1654,4935	0,0324411	
ES3PAG5	0	-0,0020042	5070	274,6	3	2	6	-91,723723	-0,0018091	
ES3EP1	170685673	0,8514242	152,3	0,6	1,5	4	6	85,142421	0,0559044	*0,090113*
ES3EP2	222319933	1,1095955	150,4	0,8	1,5	4	6	147,94607	0,0983684	

ES3EP3	437612072	2,1860562	151,9	0,6	1,5	4	6	218,60562	0,1439142	
ES3EP4	0	-0,0020042	151,5	0,5	1,5	4	6	-0,1670133	-0,0001102	
ES3EP5	34716191	0,1715768	151,9	8,1	3	2	6	231,62867	0,1524876	
ES3ELOL1	566802740	2,8320095	149,8	49,1	3	2	6	23175,278	15,470813	*15,32929*
ES3ELOL2	599128364	2,9936377	151,1	48,1	3	2	6	23998,995	15,882856	
ES3ELOL3	633689137	3,1664415	150,1	43,5	3	2	6	22956,701	15,294271	
ES3ELOL4	650715901	3,2515753	151	41,5	3	2	6	22490,063	14,894081	
ES3ELOL5	604444633	3,020219	151,3	45,4	3	2	6	22852,99	15,104422	
ES3ELOP1	348245027	1,739221	496,9	302,6	3	2	6	87714,711	17,652387	*17,75943*
ES3ELOP2	330439135	1,6501915	523,7	308,1	3	2	6	84737,334	16,180511	
ES3ELOP3	357206399	1,7840278	490,3	298,7	3	2	6	88814,852	18,11439	
ES3ELOP4	349195203	1,7439719	575,5	356,1	3	2	6	103504,73	17,985183	
ES3ELOP5	369616503	1,8460784	518	317,6	3	2	6	97719,081	18,864687	
1S3AP1	245716030	1,226576	151,5	2,7	3	2	6	551,9592	0,3643295	*0,318283*
1S3AP2	182673379	0,9113627	153	3,8	3	2	6	577,1964	0,3772525	
1S3AP3	18747793	0,0917348	152,7	21,3	3	2	6	325,65856	0,2132669	
1S3PEP1	59916705	0,2975794	508,2	2,3	3	2	6	114,07209	0,0224463	*0,018126*
1S3PEP2	4344026	0,019716	499,1	21,1	3	2	6	69,334495	0,0138919	
1S3PEP3	58384547	0,2899186	508,9	1,9	3	2	6	91,807549	0,0180404	
1S3PAP1	30878822	0,15239	490,6	13,5	3	2	6	342,87739	0,0698894	*0,071681*
1S3PAP2	30510134	0,1505465	556,5	17,4	3	2	6	436,58488	0,0784519	
1S3PAP3	164452965	0,8202607	491,9	2,4	3	2	6	328,10427	0,0667014	
1S3ELOL1	484847703	2,4222344	150,2	61	3	2	6	24626,049	16,395506	*18,99758*
1S3ELOL2	448837814	2,2421849	151,2	62	3	2	6	23169,244	15,323574	
1S3ELOL3	752104936	3,7585205	150,2	60,6	3	2	6	37961,057	25,273673	
1S3ELOP1	462078738	2,3083895	515,5	259,4	3	2	6	99799,374	19,359723	*19,82505*
1S3ELOP2	529881321	2,6474024	491	251,7	3	2	6	111058,53	22,618846	
1S3ELOP3	342972368	1,7128577	512	313,8	3	2	6	89582,457	17,496574	
1S3PR1	4731224	0,021652	151,6	3,5	3	2	6	12,63031	0,0083313	*0,049594*
1S3PR2	19613732	0,0960645	153,5	3,9	3	2	6	62,441925	0,0406788	
1S3PR3	88029559	0,4381436	153,7	2,1	3	2	6	153,35027	0,0997725	
2S3AP1	56782902	0,2819104	152	9,9	3	2	6	465,15208	0,3060211	*0,401887*
2S3AP2	153937013	0,7676809	150	5,6	3	2	6	716,50218	0,4776681	
2S3AP3	136939179	0,6826917	151	5,6	3	2	6	637,17895	0,4219728	
2S3PEP1	98015108	0,4880714	517	1,5	3	2	6	122,01785	0,0236011	*0,029982*
2S3PEP2	5820196	0,0270968	512,7	30	3	2	6	135,4841	0,0264256	
2S3PEP3	147916123	0,7375765	523,5	1,7	3	2	6	208,98	0,0399198	
2S3PAP1	2135498	0,0086733	503,1	87,2	3	2	6	126,0524	0,0250551	*0,030926*
2S3PAP2	2712217	0,0115569	493,2	92,3	3	2	6	177,78403	0,036047	
2S3PAP3	2716290	0,0115773	499,5	82	3	2	6	158,22296	0,0316763	
2S3ELOL1	281627938	1,4061355	151,3	55,4	3	2	6	12983,318	8,5811752	*10,84577*

2S3ELOL2	592313363	2,9595627	151	42,6	3	2	6	21012,895	13,915824	
2S3ELOL3	399289476	1,9944432	151,3	45,7	3	2	6	15191,009	10,040323	
2S3ELOP1	276793212	1,3819619	510	322,7	3	2	6	74326,518	14,573827	*14,75323*
2S3ELOP2	275779182	1,3768918	505	320,4	3	2	6	73526,019	14,559608	
2S3ELOP3	280301407	1,3995029	502,7	326	3	2	6	76039,656	15,126249	
2S3PR1	291018316	1,4530874	150	0,5	1,5	4	6	121,09062	0,0807271	*0,065951*
2S3PR2	41207680	0,2040342	152,4	1,9	1,5	4	6	64,610843	0,0423956	
2S3PR3	75129711	0,3736444	150	1,8	1,5	4	6	112,09332	0,0747289	
4S3AP1	51122003	0,2536059	152,2	8	3	2	6	338,14114	0,2221689	*0,162066*
4S3AP2	14684157	0,0714166	152,8	18,1	3	2	6	215,44015	0,1409949	
4S3AP3	9419297	0,0450923	152,1	24,9	3	2	6	187,13315	0,123033	
4S3PEP1	0	-0,0020042	512,9	1,7	1,5	4	6	-0,5678453	-0,0001107	*-0,00013*
4S3PEP2	0	-0,0020042	518,8	2,7	1,5	4	6	-0,901872	-0,0001738	
4S3PEP3	0	-0,0020042	501,2	1,5	1,5	4	6	-0,50104	-1,00E-04	
4S3PAP1	1401335	0,0050025	530,4	355,8	3	2	6	296,64914	0,0559293	*0,057828*
4S3PAP2	1608556	0,0060386	532	335,5	3	2	6	337,6595	0,0634698	
4S3PAP3	1388633	0,004939	518,1	340,4	3	2	6	280,20622	0,0540834	
4S3PR1	151346451	0,7547281	153,3	1,5	1,5	4	6	188,68202	0,1230803	*0,086578*
4S3PR2	86778926	0,4318905	151,3	2,1	1,5	4	6	151,16166	0,0999086	
4S3PR3	3100502	0,0134984	150	24,5	3	2	6	55,118263	0,0367455	

Table 25. Quantification of SQ by GC-MS in all samples collected throughout the olive (kernel) oil refining process. In color the samples containing high SQ amounts.

After comparison of the results listed in the above table for the first lot of olive and olive kernel oil (5S3 and ES3 respectively), it was evident that in all cases the samples analyzed, whether related to olive or olive kernel oil, the most important quantities of SQ are detected in samples of free fatty acids (FFA) that occur during deodorization. Both the oily phase and the paste contain high amounts of SQ, while the paste appears to contain slightly larger amounts of SQ than the oil phase. Specifically, the FFA are concentrated in SQ in quantities ranging between 10 and 20%. In contrast, samples of any other origin contain less than 1% squalene. On the other hand, a close watch on the variation of SQ content in the oils during the process, from the starting (unprocessed) to the finished (refined) product, reveals that during the stage of neutralization, little variation in SQ exists. For instance for lot 5S3 the SQ content is practically the same and is 0.101 and 0.009% respectively,

probably because no SQ is removed and the margarins and soaps that occur are not abundant in the unprocessed oil. During decoloration there is a significant augmentation of SQ, from 0.009% to 0.274%, probably because the unprocessed olive kernel oil contains large amounts colorants that participate a lot in the mass of the oil. Thus, while no SQ is removed during this step, the extensive removal of colorants leads to an "enrichment" of the intermediate neutralized and decolorized oil In SQ. Nevertheless, during the deodorization step large SQ removal occurs through distillation and thus on one hand the OODD (biphasic deodorizer by-product, analyzed separately by phase, 5S3ELOL and 5S3ELOP respectively) contains large amounts of SQ (12.685 and 13.271% respectively), while the refined oil occurring after this step (5S3PR) is poorer than the intermediate and also than the unprocessed oil, containing only 0.008% SQ. Similar results and conclusions arise also from olive oil analysis (lot ES3). Thus, the unprocessed oil (ES3AE) contains 0.311% SQ while the neutralized olive oil (ES3EXE) contains 0.355% SQ. The decolorized olive oil (ES3EAX) contains 0.338% SQ and the final refined olive oil 0.090% SQ. This shows that in this case the decolorization has not very important yield and does not lead to a temporary enrichment in SQ, while the losses in SQ occur again during deodorization. This is confirmed by the fact that the relevant biphasic by-product (ES3ELOL and ES3ELOP) is rich in SQ, containing 15.329 and 17.759% SQ respectively.

1.3.5) Supercritical CO2 extraction for the enrichment of SQ in the most suitable material.

As the free fatty acids-containing by-product (code ELOL and ELOP) was proved to be by far the richest in SQ, containing already among 10 and 20% of SQ, this was chosen as the material to proceed with the study. Although there were some differences between the two phases (oil and paste) that it presented, those were not very significant and it was decided that a simple homogenization would be performed before any treatment and the material would be treated as one, from now and on ELO. Indeed, for the fractionation of ELO with supercritical CO2, two types of experiences have been designed, more specifically:

- First, the idea was to perform a "pressure-wise" fractionation of the starting material. Thus, 130g of the richest in SQ by-product (OODD) were mixed with 20g of cellulose into the 1lt extractor basket and were extracted by means of supercritical CO2 by gradually raising the pressure and fractionating (80,110,180 and 290 bar) thus the extract. Samples were taken also from the ELO inside the basket after the completion of each extraction step and before raising the extraction pressure. This was achieved by isolating the extractor, eliminating pressure, taking sample for analysis and raising the pressure to the next level for the fractionation to proceed. The temperature of the extraction was kept constant at 45°C. Totally 8 fractions were collected and were analyzed along with the starting material (R0), 3 intermediate residues (R1-R3) and the final residue (R4) for their content in SQ. The Table below tabulates the procedure progress and results.

Extraction Parameters													
			Extractor 1		Extractor 2		Separators		Co-solvent	Ratio	Weight (g)	Code	SQ content (%)
Time	Flow rate (kg/h)	CO2 Temperature (°C)	Pressure (bar)	Temperature (°C)	Pressure (bar)	Temperature (°C)	Pressure (bar)	Temperature (°C)					
14:40	10	37	0	40	-	-	0	50	-	0		R0	15.86
14:50	10	45	80	45	-	-	50	50	-	0			
15:20	10	45	80	45	-	-	50	50	-	38.5	0.94	F1	9.35
15:50	10	45	80	45	-	-	50	50	-	77	0.07	F2	9.19
15:50	10	45	0	45	-	-	50	50	-	77	-	R1	11.34
16:35	10	46	110	45	-	-	50	50	-	154	10.02	F3	4.32
17:05	10	47	110	45	-	-	50	50	-	292.5	5.91	F4	3.49
17:35	10	47	110	45	-	-	50	50	-	331	4.90	F5	5.47
17:35	0	47	0	45	-	-	50	50	-	331	-	R2	12.70
18:20	10	47	180	45	-	-	50	50	-	369.5	47.51	F6	15.04
18:50	10	47	180	45	-	-	50	50	-	408	13.23	F7	10.85
18:50	0	47	0	45	-	-	50	50	-	408	-	R3	11.42
20:20	10	48	290	45	-	-	50	50	-	523.5	20.70	F8	13.13
20:20	10	48	0	45	-	-	0	50	-	523.5	11.18	R4	0.08

Table 26. Supercritical CO2 fractionation of olive kernel oil OODD: 1st trial

As it can be seen from the Table above, no succesfull enrichment was achieved during this first experiment. The richest fraction (F6) only had an SQ content slightly inferior than the starting material (15.04% vs 15.86%), while this fraction accumulated the most important quantity of SQ (7.15g) out of the total quantity of SQ extracted from the OODD (12.40g). It was assessed as an unsuccesfull experience thus.

- After these results a second process was designed for performing the "pressure-wise" fractionation in 3 steps, introducing a co-solvent (EtOH): At 120 bar with the addition of 5% EtOH as a co-solvent, at 290 bar without co-solvent and at 290 bar with 5% EtOH as a co-solvent. The temperature of the extraction was kept constant again at 45°C. 150g of OODD along with 30g of cellulose were placed in the extractor. In total 5 fractions were collected. The Table below tabulates the procedure progress and results.

Extraction Parameters													
			Extractor 1		Extractor 2		Separators		Co-solvent	Ratio	Weight (g)	Code	SQ content (%)
Time	Flow rate (kg/h)	CO2 Temperature (°C)	Pressure (bar)	Temperature (°C)	Pressure (bar)	Temperature (°C)	Pressure (bar)	Temperature (°C)	EtOH %				
14:20	10	45	120	45	-	-	50	50	5	0	-	R0	22.14
15:50	10	45	120	45	-	-	50	50	5	100	20.15	F1	12.08
16:00	10	48	290	45	-	-	50	50	-	100	-		
16:45	10	48	290	45	-	-	50	50	-	150	22.20	F2	31.85
17:30	10	49	290	45	-	-	50	50	-	200	25.51	F3	32.58
18:15	10	49	290	45	-	-	50	50	-	250	29.54	F4	33.41
18:25	10	48	290	42	-	-	50	50	5	250	-		
19:10	10	49	290	42	-	-	50	50	5	300	22.48	F5	19.14
19:10	0	47	0	42	-	-	-	-	-	300	12.15	R1	0.14

Table 27. Supercritical CO2 fractionation of olive kernel oil OODD: 2nd trial

As it can be seen from the Table above, a more successful enrichment was achieved during this first experiment. The richest fractions (F2-F4 at 290 bar without EtOH) had an average SQ content approx. 2 times more than the starting material (32.61%

vs 15.86%), while this fraction accumulated the most important quantity of SQ (25.25g) out of the total quantity of SQ extracted from the OODD (31.98g).

1.3.6) Hydrostatic CCC (CPC) single-step purification of SQ from olive kernel oil deodorizer distillates (OODD)

1.3.6a) Selection of the biphasic solvent system and determination of partition coefficients

A GC-MS analysis of the staring material (OODD) was performed, in order to provide useful information for the identities of the compounds that compose it. This would serve to the optimization of the method development for CCC. Five major compounds were identified, namely methyl palmitate, ethyl palmitate, oleic acid methyl ester, oleic acid ethyl ester and squalene.

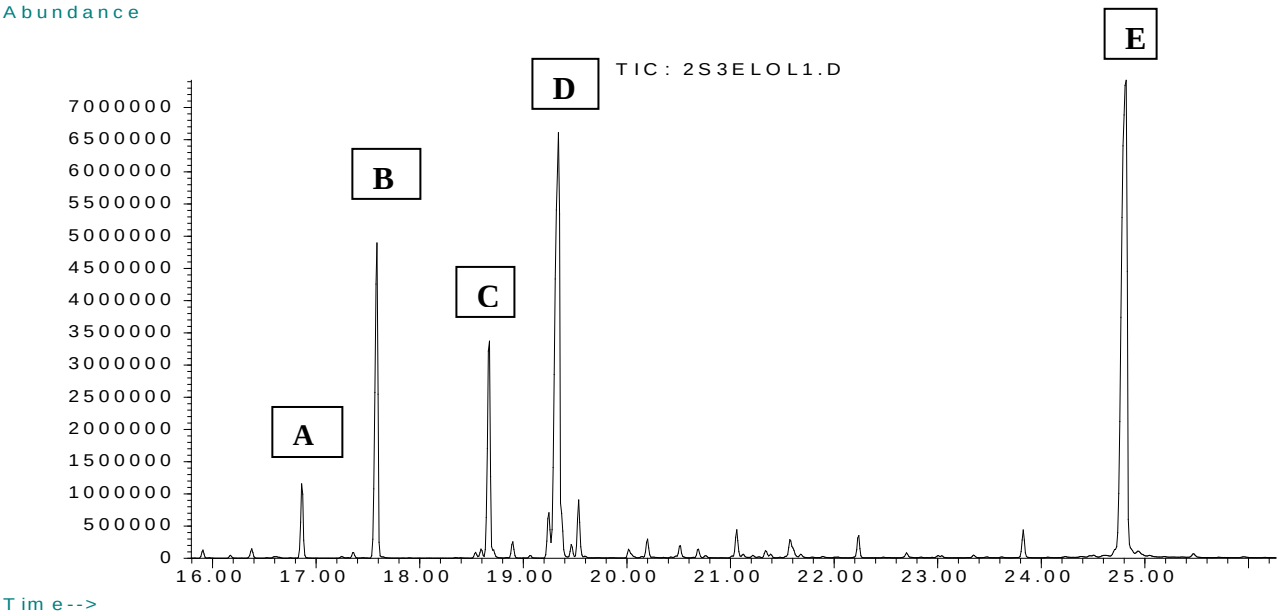


Fig.73: Methyl palmitate, B: Ethyl palmitate, C:Oleic acid methyl ester, D: Oleic acid ethyl ester, E: Squalene

The suitability of several biphasic solvent systems was first evaluated regarding the compliance to certain criteria (rapidity and quality of phase separation, volume ratio for the two phases) and subsequently by TLC, and then the solvent systems giving

the best apparent partition of SQ were further analyzed by UHPLC for the determination of partition coefficient values (K) and separation factors (α). This was performed as follows: an aliquot of OODD (10 mg) was weighed into a 10 mL glass tube, 4 mL of the pre-equilibrated biphasic solvent system were added to the sample and shaken vigorously. The mixture was centrifuged at 2000 rpm for 1 minute, and then 1 mL of each layer was taken out and evaporated to dryness. The residues were diluted in 1 mL of methanol, filtered on Nylon 0.45 µm and analyzed by UHPLC-DAD. The K value was expressed as the peak area of the target compounds in the mobile phase divided by the one in the stationary phase. In all the systems that were evaluated the mobile phase would be the upper phase and the stationary phase would be the lower phase. The separation factor (α) was expressed as the K of SQ divided by the K of another of the major compounds of the mixture (OODD) for a certain biphasic system.

Several solvent systems were proposed according to previous experience and theoretical approaches. These solvent systems were assayed for the quality and rapidity of the separation of the two phases, by implementing a simple system of visual evaluation and grading by one up to three stars these two important parameters (Table 1, systems 1–5). At a next step, the systems 1,2,3 and 5 that complied with the criteria, were analyzed (each phase separately) for the partition of SQ and the other major compounds, namely compounds A,B,C,D and E. The separation factor (α) was calculated as well (Table 2). The whole approach of the evaluation of the candidate systems was based on the work presented by Ito [14] on selecting optimum conditions for CCC separations. According to this work, a suitable K value would be $1.0 \leq K \leq 2.0$, while a suitable α value would be $0.67 \geq \alpha \geq 1.5$.

System	Composition	Separation quality	Separation time
S1	Hept/DCM/ACN 2/0.8/1.2	**	***
S2	Hept/ACN/BuOH	***	***

	1.8/1.4/0.7		
S3	Hept/cHex/DCM/ACN 1.2/0.8/0.8/1.2	***	***
S4	cHex/MeOH 2.6/1.3	*	*
S5	nHex/MeOH 2.7/1.3	***	***

Table 28. Assessment of phase separation for the biphasic systems 1-5

		S1 - UP	S1 - LOW	S2 - UP	S2 - LOW	S3 - UP	S3 - LOW	S5 - UP	S5 - LOW	TOTAL
Compound A	UHPLC Area	175438	156079	154811	80622	153463	199268	164308	127587	134960
	$k_{U/L}$	**1.124033**		**1.920208**		**0.770134**		**1.28781**		
	$\alpha=k_{SQ}/k_A$	**3.046296**		**3.202026**		**3.009907**		**4.84472**		
Compound B	UHPLC Area	113412	34595	103165	0.001	98971	45396	107055	41251	86843
	$k_{U/L}$	**3.278277**		**1.03E+08**		**2.18017**		**2.59521**		
	$\alpha=k_{SQ}/k_B$	**1.044493**		**5.96E-08**		**1.063234**		**2.40408**		
Compound C	UHPLC Area	199841	96077	155963	43990	160174	131379	154738	72746	111561
	$k_{U/L}$	**2.080009**		**3.545419**		**1.219175**		**2.1271**		
	$\alpha=k_{SQ}/k_C$	**1.646213**		**1.734225**		**1.901311**		**2.93315**		
Compound D	UHPLC Area	12102	22631	45549	31575	43678	23349	57061	16218	33569
	$k_{U/L}$	**0.534753**		**1.442565**		**1.870658**		**3.51837**		
	$\alpha=k_{SQ}/k_D$	**6.403211**		**4.262237**		**1.239152**		**1.77329**		
SQUALENE	UHPLC Area	**2688746**	785233	**2367458**	385043	**2481913**	1070699	**2462994**	394768	1511626
	$k_{U/L}$	**3.424138**		**6.148555**		**2.318031**		**6.23909**		
Compound E	UHPLC Area	45579	5599	46859	25489	54973	44579	44569	12512	31223
	$k_{U/L}$	**8.140561**		**1.838401**		**1.233159**		**3.5621**		
	$\alpha=k_{SQ}/k_E$	**0.420627**		**3.344513**		**1.87975**		**1.75152**		

Table 29. Calculation of K and α for the biphasic systems 1, 2, 3 and 5

As it can be seen in Table 1, the solvent systems S2, S3 and S5 had the best separation quality and time, while S1 was quite good and S4 was rather poor. For this reason S4 was excluded from the UHPLC quantitative analysis for the determination of the partition coefficient (K) and the separation factor (α). As it can be seen in Table 2, for all systems SQ had a K superior to 1.5. More specifically systems S1 and S3 had a more even distribution of SQ in the two phases (K values 3.424138 and 2.318031 respectively), as compared to S2 and S5 (K values 6.148555 and 6.23909 respectively). Nevertheless, for S1 compounds B and SQ presented a

separation factor that did not comply with the criteria (1.044493), while for system S3, compounds B and SQ as well as D and SQ presented a separation factor that did not comply with the criteria (1.063234 and 1.239152 respectively). On the contrary, regarding systems S2 and S5, all α values complied with the criteria. Since system S5 was already tested for SQ purification in a previous work, even if this regarded a different source -microalga- [10] it was decided to proceed to the CCC purification with system S2 (Hept/ACN/BuOH : 1.8/1.4/0.7, v/v/v).

1.3.6b) Separation process

As stated before, the preparative separations were carried out on a Kromaton (Angers, France) FCPC® instrument equipped with a rotor of 1000 mL. After filling with the stationary phase (upper phase), the rotation was set at 850 rpm (in order to keep a high "g" field and a good resolution).and the mobile phase pumped through the system at a flow-rate of 10 mL/min. With these parameters, the retention of the stationary phase (Sf) into the column was 70%. This characteristic – the higher proportion of the stationary phase – is one of the main advantages of CCC over solid-liquid chromatography, in which the accessible stationary phase represents generally 5 to 15% only of the total column volume. This larger proportion enables to increase dramatically the load capacity, which can reach up to 10% of the total column volume. Furthermore, in the case of OODD, which is a liquid mixture, there is no issue about sample solubility. Another advantage of high Sf values is that they generate better resolutions. This suggests that the scale-up of the method to the preparative centrifuge should not result in a significant loss of resolution.

Fractions (30 mL) were collected immediately after the injection of OODD, every 3 minutes. The biphasic solvent system consisting of heptane–acetonitrile–butanol (1.8:1.4:0.7, v/v/v) was selected as the most promising. The solvents were thoroughly mixed in a separating funnel at room temperature prior to use, and the two phases of each system were separated after equilibration of the mixture. OODD was diluted in an equal volume of each phase from the solvent system prior to injection. 5.0 g of OODD dissolved in a total volume of 30 mL. The total analysis time was 4 hours. The SQ fraction was eluted at approx. 60-100 min since the elution step

began with a good resolution while a fraction containing the more polar FFA and their esters (Rt~100-140 min.) was also obtained.

The CCC fractions derived from the isolation where initially evaluated using TLC analysis with sulfuric vanilin. Subsequently, a UHPLC method was developed to analyze the fractions obtained from CCC separations. Acetonitrile 100% was used without any modifiers in a simple isocratic procedure. To improve chromatographic performance, the separations were carried out at a temperature of 40°C. At this temperature and at a flow-rate of 600 µL/min, the total analysis time could be reduced to 3 minutes without losing any resolution.

In OODD analysis, as well as for the upper and lower phases of the evaluated biphasic systems, the first group of peaks, representing the more polar FFA and their esters, were detected between 0.5 and 1.1 minutes while SQ was detected between 1.37 and 1.43 minutes. The developed UHPLC-DAD method enabled to clearly separate the different groups of compounds of OODD, in a very reasonable analysis time. The CCC fractions could thus be analyzed by this way to confirm purity of the isolated SQ. As expected from the TLC analysis, the fractions 5-18 were shown to contain almost exclusively SQ (Fig. 4). Based on UHPLC-UV detection, the purity of SQ was really high, as it was above 95%.

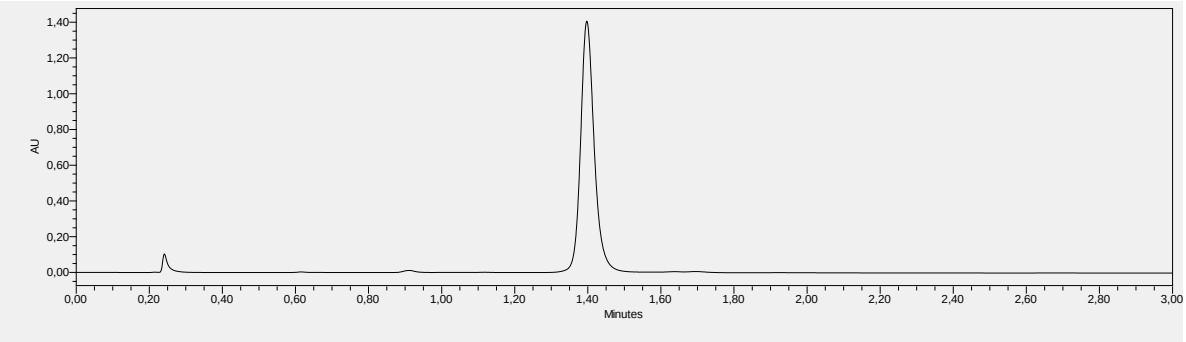


Fig.74. Purified SQ (fractions 5-18) UHPLC-DAD chromatogram at 210nm

The purest fractions containing SQ were brought together, leading to a total yield of 2413mg, i.e. 19.6% of OODD.

1.3.7) Conclusions

Due to its multiple beneficial and potential properties, squalene is a natural bioactive product of significant interest. There is a real need to produce squalene of high

quality and purity from vegetal sources in order to replace animal-origin squalene and to supply companies, in the fields of chemistry, cosmetics, nutraceuticals and pharmaceuticals.

An evaluation of all by-products that occur from olive and olive kernel oil refining industry led to the clear conclusion that the deodorizer distillates are the only by-product that contain significant amounts of SQ, making it worthwhile to develop an enrichment/purification process.

Supercritical CO2 proved to be only partially interesting for the enrichment of SQ, with the parallel use of EtOH as a co-solvent in certain steps of the process. This facilitated the fractionation of SQ by modifying polarity and solvating power of the extractant.

On the other hand, liquid-liquid partition chromatography presented significant advantages for the preparative purification of squalene in comparison with supercritical CO2. Over supercritical CO2 fractionation, CCC offered the advantage of leading higher purities due to the chromatographic nature of the separation. Countercurrent chromatography is thus a technique of choice to improve production yields. The developed method, adapted to the low polarity of olive oil deodorizer distillates, enabled in one single step to recover the target compound. Moreover, the purity of isolated squalene was particularly high, as proven by the developed UHPLC-DAD method. Therefore, the exploitation at the industrial level of the newly developed hydrostatic CCC method could provide a potent source for the rapid production of high-added value squalene from olive oil refining by-products.

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